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FRACTIONATION OF HYPOTHALAMIC TISSUE FOR PITUITARY-STIMULATING ACTIVITY[1]

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AN INTACT posterior hypothalamus appears to be essential for the release of pituitary adrenocorticotrophic hormone (ACTH) in response to stress (De Groot and Harris, 1950; Porter, 1952, 1953). The neurophysiological mechanism responsible for this release has not yet been established. However, it has been proposed by Harris (1948), Hume (1949), and others that neural stimulation may result in the formation of a hypothalamic neurohumor which ultimately provokes the secretion of ACTH by the adenohypophysis. This substance is believed to be carried to the adenohypophysis via the portal vessels of the pituitary stalk (Harris, 1948).

Previous attempts have been made to demonstrate the existence of the postulated neurohumor. Injection of material prepared by acetone extraction of *whole* bovine hypothalami has been reported to result in eosinopenia in dogs (Hume and Wittenstein, 1950) and leukopenia in immature rats (Buchanan *et al.*, 1952; Hellerstein *et al.*, 1952). In the present investigations, extracts of various areas of bovine brain were tested for their ability to stimulate ACTH release by the adenohypophysis. The results obtained suggest that the posterior hypothalamus may elaborate specifically a pituitary-stimulating factor. This substance appears to be lipide or lipoprotein in nature.

Received for publication November 17, 1953.

[1] This investigation was supported by research grants from the National Institute of Neurological Diseases and Blindness (B-575) and from the Division of Research Grants (G-3869) of the National Institutes of Health, Public Health Service.

MATERIALS AND METHODS

Preparation of Extracts

Brain cortex and hypothalamic tissue of bovine origin were collected at the slaughter house,[2] frozen immediately, and stored in the deep freeze. At the time of extraction, the frozen hypothalami were dissected[3] as shown in Figure 1. The optic tract (OT) was removed and the hypothalamus separated from anterior portions of the brain at the posterior border of the anterior commissure (AC). Separation from posterior portions of the brain was made at the posterior region of the mammillary bodies (MB). Finally, the hypothalamus was dissected away from the remainder of the brain at the hypothalamic sulcus, and divided into anterior and posterior portions as indicated by the dotted lines in Figure 1. Median eminence tissue was not present in any of the brain samples extracted.

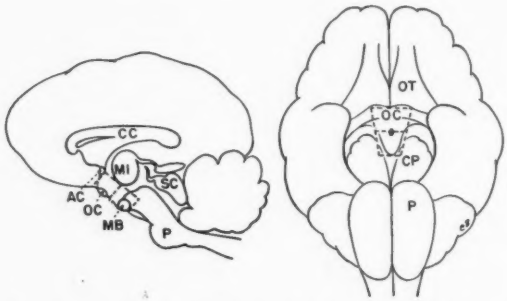

FIG. 1. *Sagittal and ventral views of bovine brain.* The following abbreviations are employed: AC, anterior commissure; CC, corpus callosum; CP, cerebral peduncle; MB, mammillary body; MI, massa intermedia; OC, optic chiasma; OT, optic tract; P, pons; SC, superior colliculus.

Two types of extracts were made from the anterior and posterior hypothalami and from the cerebral cortex (cf. Fig. 2). An aqueous extract was prepared from one aliquot of tissue, and a lipide extract from a second aliquot. The aqueous extract was subdivided by dialysis into protein and non-protein fractions. The lipide extract was further fractionated into saponifiable and non-saponifiable portions by the techniques described below.

Protein fraction. An aliquot of tissue weighing about 25 gm. was homogenized in a Waring blendor at 0° C. with 100 ml. of cold distilled water at pH 7.2. The homogenate was then dialyzed for 2 days against distilled water in the refrigerator. More prolonged dialysis was found to result in a loss of pituitary-stimulating activity from the protein fraction prepared from the posterior hypothalamus (see below). The dialyzed material was lyophilized and stored in a desiccator in the deep freeze until used. It was dissolved in saline for assay. The yield of the protein fraction was approximately 4 gm. per 25 gm. of bovine hyopthalamus or cerebral cortex.

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[2] Courtesy of the Alpha-Beta Packing House, Huntington Beach, California.

[3] We are indebted to Dr. Robert W. Porter, Veterans Administration Hospital, Long Beach, California, for his help in dissection of bovine hypothalami.

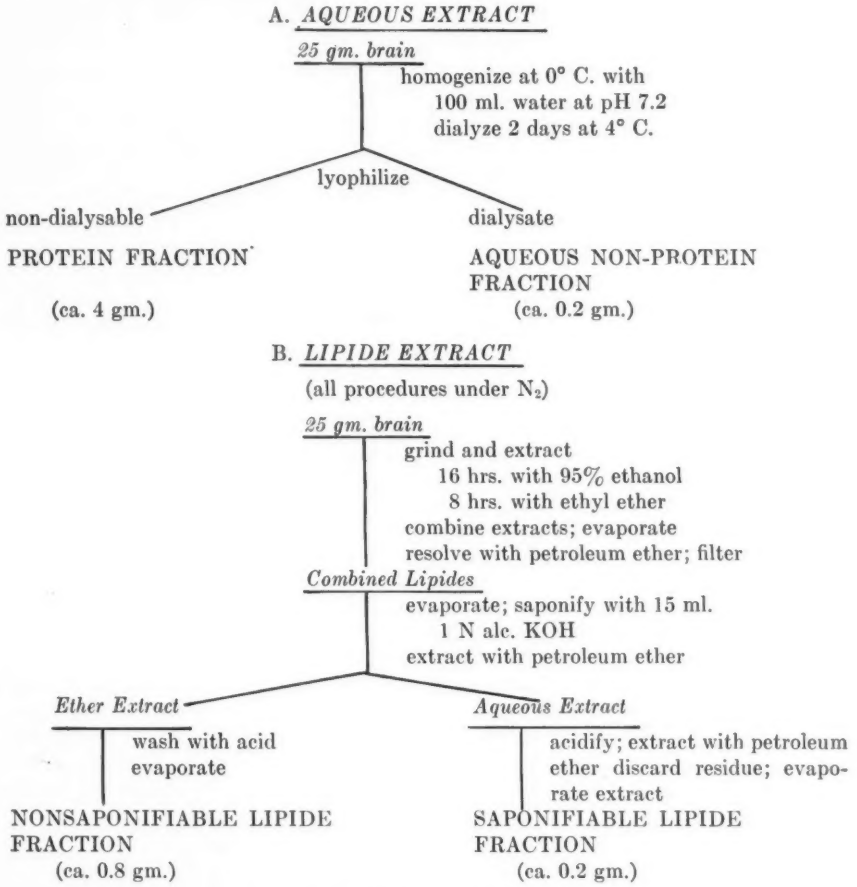

FIG. 2. *Fractionation of bovine brain.*

Aqueous non-protein fraction. This fraction was prepared by lyophilization of the dialysate obtained from the aqueous extract. It appeared to be protein-free as judged by the lack of material precipitable by trichloroacetic acid. This fraction was also stored in a desiccator in the deep freeze. It was dissolved in saline immediately prior to assay. The yield was approximately 0.2 gm. per 25 gm. of tissue, irrespective of the portion of bovine brain extracted.

Lipide extracts. A second aliquot of frozen brain tissue weighing approximately 25 gm. was ground in a chilled mortar with sand and several portions of 95% ethyl alcohol. This mixture was then transferred to a Soxhlet thimble and extracted for 16 hours with 95% ethyl alcohol, followed by 8 hours with ethyl ether. It was found necessary to carry out this extraction and all subsequent manipulations of the lipide fraction under nitrogen to prevent inactivation of the non-saponifiable lipide substance of the posterior hypothalamus (see below). The alcohol and ether extracts were combined and evaporated

under a stream of nitrogen at room temperature. The residue was resolved with warm petroleum ether and filtered through fat-free Whatman No. 43 filter paper. This filtrate and all other petroleum ether extracts were evaporated to dryness under nitrogen at room temperature.

The dried extract was saponified under nitrogen on the steam bath with 15 ml. of 1 N alcoholic KOH, and then extracted with petroleum ether. The non-saponifiable lipide fraction, present in the petroleum ether extract, was washed with water made acid to Congo Red and then evaporated to dryness. The aqueous extract containing saponified lipide was acidified to Congo Red, heated several minutes on a steam bath, and extracted with petroleum ether. This extract was also evaporated to dryness. Both lipide fractions were desiccated and stored in the deep freeze. They were assayed dissolved in sesame oil. The non-saponifiable material in each instance weighed approximately 0.8 gm. per 25 mg. of bovine brain tissue. The yield of the saponifiable fraction was about 0.2 gm. per 25 gm.

Assays

Intact, adult male Wistar rats[4] were employed in all assays, except as otherwise indicated. Three different procedures were used for measuring the biological activity of the extracts. These were blood eosinophil depression, adrenal ascorbic acid decline, and elevation of the Q_{O_2} of anterior pituitary tissue *in vitro*.

Eosinophil counts were made on freely-flowing blood obtained from the tail vein at 0, 1, and 4 hours after the intraperitoneal injection of 0.1 ml. of aqueous or lipide extracts in the appropriate vehicles into rats weighing 175–250 gm. Determinations were made by the method of Randolph (1944). Rats were used only twice for this purpose, and at least two weeks were permitted to elapse before the second assay.

Routine assays of adrenal ascorbic acid depletion were carried out in intact Wistar rats weighing approximately 200–300 gm. Active fractions were further tested in intact and hypophysectomized male Sprague-Dawley rats weighing about 200 gm.[5] Ascorbic acid was measured in both adrenal glands 2 hours after intraperitoneal injection of the various extracts or the vehicles in which they were dissolved, in amounts equivalent to 0.1 ml. per 100 gm. body weight. The methods described by Sayers, Sayers, and Woodbury (1948) were employed for the extraction and determination of adrenal ascorbic acid.

The capacity of hypothalamic extracts to elevate the respiration of anterior pituitary tissue was studied using the technique described by Roberts and Keller (1953). The incubation medium was fortified with glucose in all instances. The material to be assayed was tipped in from the side-arm of the Warburg flask after incubation had proceeded for 1 hour. During this time the "basal" level of oxygen consumption was determined. The percentage change in respiration during the second hour of incubation was taken as a measure of the pituitary-stimulating activity of the added material.[6]

RESULTS

1. *Influence of bovine brain extracts on blood eosinophil levels*

Aqueous non-protein fractions of all portions of the bovine brain assayed

[4] Obtained from Pacific Animal Farms, Los Angeles, California.

[5] Obtained from Hormone Assay Laboratories, Inc., Chicago, Illinois.

[6] *In vitro* studies of the pituitary-stimulating activity of hypothalamic extracts were carried out by Mrs. Martha R. Keller.

produced a significant eosinopenia in the adult rat. Figure 3 indicates that 40 μg. of this fraction obtained from either anterior or posterior hypothalamus was capable of eliciting a 50% decline in blood eosinophils 4 hours after intraperitoneal administration. This was the same quantitative response as that evoked by 40 μg. of epinephrine (1:1000, U.S.P.). The nonspecific origin of the aqueous non-protein fraction was established by the observation that comparable extracts of the brain cortex had a similar action (not shown). Saline, the vehicle employed for these injections, was without significant effect on blood eosinophil levels in the rat (Fig. 3).

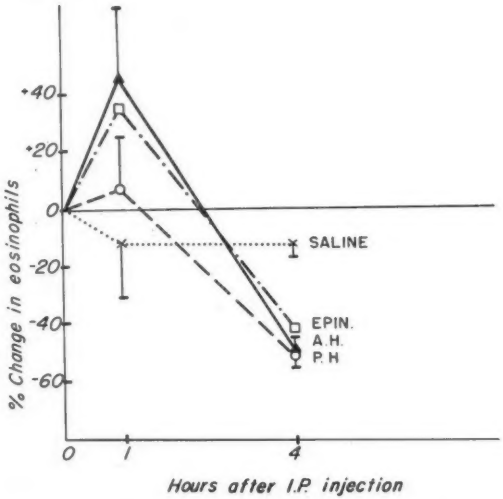

FIG. 3. *Influence of the aqueous non-protein fractions of bovine hypothalamus on blood eosinophil levels in the rat.* The change in blood eosinophils is depicted as the mean per cent change from control levels ± the standard error (T-shaped symbols) after intraperitoneal injection into rats of 0.1 ml. of saline alone, or this amount of vehicle containing 40 μg. of epinephrine solution (EPIN.), or 40 μg. of the aqueous non-protein fraction of anterior (A.H.) or posterior (P.H.) hypothalamus. Each point represents the average of 6 to 10 determinations in individual animals. The P value for the differences between the control group injected with saline alone and each of the other groups was less than 0.01.

In contrast to the lack of specificity of the aqueous non-protein fraction, protein and non-saponifiable lipide fractions which were highly active in evoking the eosinopenic response in rats could be obtained only from the posterior hypothalamus (Figs. 4 and 5). The amount of protein material (Fig. 4) required to produce a 50% drop in blood eosinophils under the conditions of the assay was approximately 5 times as much as the weight

of non-saponifiable lipide (Fig. 5) necessary to elicit the same response (i.e., 630 μg. protein vs. 125 μg. lipide). In both cases, however, the active dose represented an equivalent amount of brain tissue (ca. 4 mg.).

The protein and non-saponifiable lipide fractions of other portions of bovine brain were without significant effect on blood eosinophil levels, even

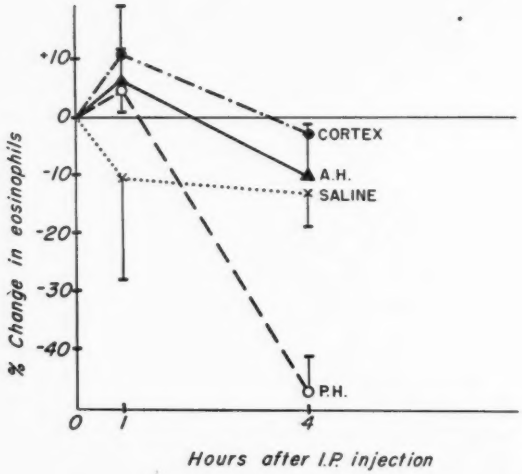

FIG. 4. *Influence of the protein fractions of bovine brain on blood eosinophil levels in the rat.* Each point represents the average ± S.E. of 6 to 13 determinations in individual rats after intraperitoneal injection of 0.1 ml. of saline or 630 μg. (in 0.1 ml. saline) of the protein fractions of those portions of the brain designated by the appropriate symbols. The P value between the control group and the "P.H." group was less than 0.01.

at twice the doses found to be active for comparable fractions obtained from the posterior hypothalamus. Saponifiable lipide material prepared from anterior or posterior hypothalamus, or from cerebral cortex, did not produce eosinopenia in amounts as large as 250 μg. per animal.

Cholesterol, the major constituent of the non-saponifiable material extracted from brain tissue, was incapable of producing eosinopenia at a dose level of 50 μg. in 0.1 ml. sesame oil.

2. *Influence of bovine hypothalamic extracts on adrenal ascorbic acid levels*

Eosinophil-depressing materials of hypothalamic origin were tested for their capacity to deplete adrenal ascorbic acid when administered to the rat. The relatively high specificity of the latter index of ACTH release seems well established (cf. Sayers, 1950).

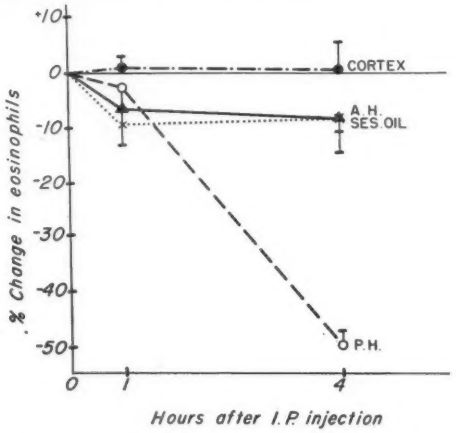

FIG. 5. *Influence of the non-saponifiable lipide fractions of bovine brain on blood eosinophil levels in the rat.* Each point represents the average ± S.E. of 6 to 14 determinations in individual rats after intraperitoneal injection of 0.1 ml. of seasame oil alone, or this amount of vehicle containing 125 μg. of the non-saponifiable lipide fraction of different portions of the brain. The P value between the control group injected with sesame oil alone and the "P.H." group was less than 0.01.

Table 1 reveals that the protein and non-saponifiable lipide fractions of the posterior hypothalamus produced in intact Wistar rats significant declines (about 30%) in adrenal ascorbic acid at dosage levels which were equivalent on a brain weight basis (i.e., 5 mg. protein per 100 gm. body weight or 1.0 mg. lipide per 100 gm. body weight. The responses to these active fractions were of similar magnitude in intact Sprague-Dawley rats (not shown in the table). Comparable effects could not be elicited upon injection of the active fractions into hypophysectomized Sprague-Dawley rats, 4 to 5 days after operation (Table 1).

The aqueous non-protein material of the posterior hypothalamus was inactive when assayed in intact rats at dose levels as high as 80 μg. per 100 gm. body weight, even though one-fourth this amount of epinephrine was capable of producing a 30% decline in adrenal ascorbic acid. All fractions prepared from the anterior hypothalamus were without significant effect on this criterion of pituitary stimulation at similar dose levels. The vehicles employed in the assay procedures, saline and sesame oil, produced no significant change in adrenal ascorbic acid levels when injected intraperitoneally into intact rats in amounts equivalent to 0.1 ml. per 100 gm. body weight (Table 1). Cholesterol was also inactive at a dosage level of 500 μg. (in 0.1 ml. sesame oil) per 100 gm. body weight.

TABLE 1. THE INFLUENCE OF EXTRACTS OF BOVINE HYPOTHALAMUS ON ADRENAL ASCORBIC ACID IN INTACT AND HYPOPHYSECTOMIZED RATS

Material Assayed[1]	Dose (mg./100 gm. body wt.)	Ascorbic Acid[2] (mg./100 gm. adrenal)	
		Intact	Hypophysectomized
None	—	435 ± 14 (15)	
Saline (control)	—	431 ± 14 (34)	400 ± 14 (6)
Epinephrine	0.02	*309 ± 9 (14)	
Aqueous			
A.H.	0.08	419 ± 34 (6)	
P.H.	0.08	390 ± 22 (6)	
Protein			
A.H.	5.0	447 ± 19 (9)	
P.H.	5.0	*305 ± 17 (9)	357 ± 9 (10)
Sesame oil (control)	—	485 ± 9 (26)	362 ± 16 (6)
Non-sap. lipide			
A.H.	1.0	449 ± 18 (8)	
P.H.	1.0	†338 ± 26 (7)	
P.H.	2.0	†300 ± 29 (5)	415 ± 16 (11)
Cholesterol	0.5	458 ± 13 (6)	

[1] All materials were injected in 0.9% saline, except the non-saponifiable lipide fractions and cholesterol which were administered in sesame oil.
[2] Average ± standard error of the mean. Number of animals shown in parentheses.
* $P < 0.01$, for the difference between this group and the saline controls.
† $P < 0.01$, for the difference between this group and the sesame oil controls.

3. *Influence of bovine hypothalamic extracts on respiration of anterior pituitary tissue in vitro*

It was hoped that it would be possible to utilize a simple *in vitro* test for the assay of pituitary-stimulating activity in the brain fractions. The observation had previously been made that epinephrine added *in vitro* stimulated the respiration of surviving adenohypophyseal tissue (Keller and Roberts, 1953). This action, however, appeared to be limited to adrenergic substances and, in the present experiments, could be duplicated only by the aqueous non-protein fraction of the hypothalamus.

Figure 6 shows that the Q_{O_2} of rat anterior pituitary tissue was markedly increased by the *in vitro* addition of 4 μg. of the aqueous non-protein fraction of either anterior or posterior bovine hypothalamus. In the concentrations used, lipide and protein fractions of the hypothalamus appeared to be without significant effect on this index of hypophyseal stimulation.

DISCUSSION

In the present experiments, at least 2 different materials could be extracted from bovine brain which were capable of causing eosinopenia after intraperitoneal administration to the rat. Of the 4 fractions prepared (protein, aqueous non-protein, saponifiable lipide, and non-saponifiable lipide) only the aqueous non-protein fraction contained pituitary-stimulating activity irrespective of the area of bovine brain extracted. This fraction was potent in causing eosinopenia in rats and an increase in oxygen consump-

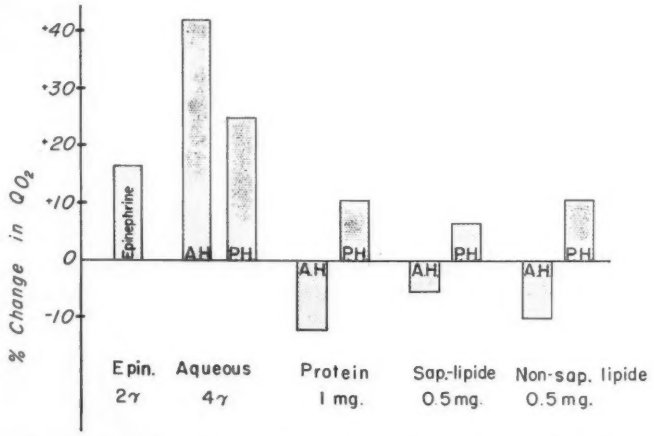

FIG. 6. *Influence of epinephrine and various fractions of bovine hypothalamus on the respiration of rat anterior pituitary tissue.* The results are expressed as the per cent change in Q_{O_2} during the second hour of incubation in micro Warburg flasks as a result of the addition from the side-arm of the substances indicated. All materials to be assayed were added in 0.05 ml. of medium with the exception of the lipide fractions which were dissolved in 0.02 ml. of 50% ethanol. Each value is the average of 4 experiments.

tion by rat adenohypophyseal tissue *in vitro*. The similarity in these responses to those obtained under identical circumstances with epinephrine suggests that the active material present in the aqueous non-protein material may be adrenergic in nature. However, unlike epinephrine (Long and Fry, 1945; Gershberg *et al.*, 1950), this fraction was incapable of provoking adrenal ascorbic acid depletion in the rat, at least under the experimental conditions employed. It is of interest to note that a water-soluble substance, "encephalin," which appeared to be sympathomimetic in action, was found by Raab (1948) to be generally distributed throughout the brain.

The capacity to provoke the discharge of pituitary ACTH seemed to be present in protein and non-saponifiable lipide fractions of the posterior hypothalamus, but not in comparable fractions prepared from other portions of bovine brain. The dosages of the protein and non-saponifiable lipide fractions required to produce a similar depression of blood eosinophils or adrenal ascorbic acid in the intact rat were found to represent equivalent amounts of hypothalamic tissue. This observation suggests that the active hypothalamic factor may occur as a lipide-protein complex in its natural state. The intact lipoprotein might be expected to be present in the protein fraction. Preliminary observations appear to substantiate these conclusions. Thus, prolonged dialysis or cold ethanol extraction of the protein fraction prepared from the posterior hypothalamus were found to remove biological activity. The chemical nature of the active non-saponifiable lip-

ide material is unknown at this time, but it is not cholesterol. Its physiological rôle, and its relationship to the postulated hypothalamic neurohumor, still remain to be clarified. It is anticipated that isolation of a more purified "hypothalamic hormone," and its biochemical characterization, may make this possible.

SUMMARY

At least two materials present in extracts of bovine hypothalamus appeared to be capable of stimulating pituitary release of ACTH as judged by their ability to cause eosinopenia or a decline in adrenal ascorbic acid after intraperitoneal administration to intact rats. One factor was a water-soluble, non-protein substance present in all parts of the brain analyzed. The second factor, which was lipide or lipoprotein in nature, was present only in extracts of the posterior hypothalamus and required the presence of an intact pituitary gland for its action on adrenal ascorbic acid levels. The lipide portion of this active specific substance was non-saponifiable but not cholesterol. This lipoidal substance may represent, in crude from, the natural neurohumor presumed involved in the stress-induced release of ACTH by the adenohypophysis.

Acknowledgments

Grateful acknowledgment is extended to Miss Antoinette Demming, Mrs. Beverly Lilly, and Miss Irene Rask for their technical assistance in all procedures and to Messrs. Charles Bridgman and Timothy Dodge for the preparation of the figures.

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CYTOLOGICAL DEMONSTRATION OF NORADRENALINE IN THE SUPRARENAL MEDULLA UNDER CONDITIONS OF VARIED SECRETORY ACTIVITY

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OXIDATION of noradrenaline *in vitro* with potassium iodate gives a selective pigment formation, making possible differentiation from adrenaline (Hillarp and Hökfelt, 1953). When applied cytologically to the adrenal medulla this reaction of pigment formation suggested that noradrenaline is stored within special medullary cells. These investigations, however, did not exclude the possibility that the "noradrenaline cells" are non-specific, representing only varying degrees of secretory activity in the medulla.

Using the above method for cytological demonstration of noradrenaline the adrenal medulla has now been studied under different conditions of secretory activity.

MATERIALS AND METHODS

Only adult cats and rats were used.—In order to obtain *minimal secretory activity* from the suprarenal medulla denervation was performed. In four cats all nerves innervating the medulla were cut close to the adrenal gland. In the rat (21 animals) by an intra-abdominal approach the great splanchnic nerve was cut immediately cranial to its exit from the thorax; in this way the adrenal gland of the rat is totally denervated (Hillarp, 1946).

In both the cat and rat denervation was performed of the left suprarenal gland only; the right served as a control. The animals were kept for 2–4 weeks after operation.

Increased secretory activity was obtained (8 cats and 16 rats) by insulin induced hypoglycemia as described for the cat by Hökfelt (1953) and for the rat by Hillarp, Hökfelt and Nilson (1954).

For the cytological demonstration of noradrenaline in the suprarenal medulla the above mentioned oxidation with potassium iodate was used and the simultaneous presence of adrenaline and noradrenaline was demonstrated by oxidation with potassium bichromate; both methods according to Hillarp and Hökfelt (1953).

For quantitative assay of the catechol amines the suprarenal glands were extracted with acid ethanol and estimated colorimetrically according to Euler and Hamberg (1949) and biologically according to Euler (1949).

Received for publication December 14, 1953.

RESULTS

As previously described (Hillarp and Hökfelt, 1953) the application of the oxidative procedure with potassium iodate to the normal suprarenal gland of the cat results in an intense pigment formation, localized within well delineated areas of cell complexes, regularly amounting to 30–40% of the medullary cells. Following oxidation with potassium bichromate there is a marked pigment formation in all medullary cells. In the rat potassium iodate gives pigment formation in 5–15% of the medullary cells while all cells react with potassium bichromate.

Pigment formation within the cells following oxidation with postassium iodate appears to selectively demonstrate noradrenaline, while after oxidation with potassium bichromate pigment formation is due to either adrenaline or noradrenaline or both.

1. *The adrenal medulla in minimal secretory activity*

In spite of secretory inactivity for two to four weeks following denervation of the adrenal gland no change of the cytological picture obtained by any of the two oxidation procedures used could be observed either in the cat or in the rat when compared to the non-denervated adrenal medulla. Thus, the well-delineated sections of adrenal medullary cell complexes (30–40% of the adrenal medulla) which by oxidation with potassium iodate display an intense pigment formation and which appear in the normal adrenal medulla of the cat, were also visible after denervation (Fig. 1a). The remaining cells showed no or very slight reaction as in the normal adrenal medulla. On oxidation with potassium bichromate all cells—as under normal conditions—responded by intense pigment formation (Fig. 1b).

Following adrenal denervation in the rat there was also no change of the reactions of pigment formation.

In unpublished experiments under identical conditions Euler and Hökfelt estimated quantitatively (colorimetrically and biologically) the catechol content of the denervated adrenal gland and found it to be equal to that of the intact suprarenal gland (cat and rat).

2. *The adrenal medulla in increased secretory activity*

Selective depletion of the adrenaline content of the suprarenal gland, without any decrease of the noradrenaline can be produced in the cat (Hökfelt, 1953) and the rat (Hökfelt, 1951) by insulin induced hypoglycemia provided the hypoglycemia is not severe enough to cause convulsions and coma. If such symptoms appear they can be counteracted by subcutaneous administration of glucose. This technique permits an investigation of the cytological distribution of noradrenaline and the specificity of the "noradrenaline cells."

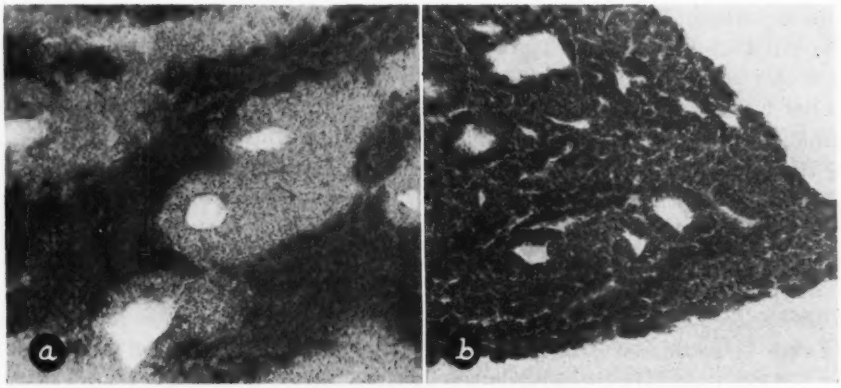

FIG. 1. Cytological appearance of the suprarenal medulla of the cat two weeks after denervation. a) Oxidation with potassium iodate caused pigment formation only within distinct, well delineated areas of cell complexes. b) Oxidation with potassium bichromate was followed by pigment formation in all medullary cells.—The same cytological picture were seen in the *non*-denervated medulla after oxidation with the respective reagents. ×85.

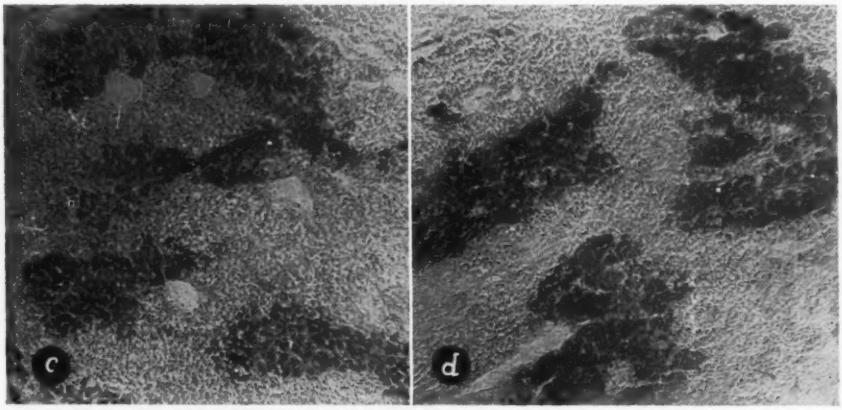

FIG. 1. Cytological appearance of the suprarenal medulla in the cat after treatment with insulin (cat No. XII, table 1). c) Oxidation with potassium iodate was followed by intense pigment formation localized within well delineated areas of cell complexes; the same picture was seen in both the normal and the denervated adrenal medulla. d) Oxidation with potassium bichromate—like potassium iodate—now caused pigment formation only within special areas of cell complexes. This was in contrast to the picture obtained in the normal and denervated adrenal medulla (cf. fig. 1b). ×85.

To obtain the different cytological pictures corresponding to varying degrees of secretory activity and consequently various degrees of adrenaline depletion, different doses of insulin were used and allowed to act

under different lengths of time (2–8 I.U. per kg. body weight, 6–21 hours for the cat and 10 I.U. per 100 gm. body weight, 2–9 hours for the rat). In the cat one adrenal gland was taken for cytological examination, while the other was extracted for quantitative estimation of the amounts of adrenaline and noradrenaline remaining after stimulation with insulin.

As seen in table 1 the adrenaline content of the normal adrenal gland was 1.14 μg. $\pm$0.139 per mg. tissue in the male cat (Nos. I to VIII) and 0.55 μg. $\pm$0.028 in the female cat (Nos. XIV to XIX); for noradrenaline the corresponding values were 0.96 μg. $\pm$0.119 and 0.55 μg. $\pm$0.071 respectively.

After treatment with insulin cats Nos. IX to XI, XXI and XXII (Table 1) presented a marked decrease in the adrenaline content when compared to the controls, while the noradrenaline values were still of nor-

TABLE 1. ADRENALINE AND NORADRENALINE CONTENT OF THE SUPRARENAL GLAND IN THE CAT FOLLOWING THE INJECTION OF INSULIN. THE FIGURES ARE DIVIDED INTO TWO GROUPS ACCORDING TO SEX

	No.	Adrenaline μg./mg. tissue	Noradrenaline μg./mg. tissue	Adrenaline in per cent of both catechols
		MALE		
Controls	I–VIII	1.14 $\pm$ 0.139	0.96 $\pm$ 0.119	54.4 $\pm$ 2.21
Insulin injected	IX	0.61	1.06	36.5
Insulin injected	X	0.57	1.35	29.7
Insulin injected	XI	0.55	0.87	38.7
Insulin injected	XII	0.35	1.09	24.3
Insulin injected	XIII	0.33	0.98	25.2
		FEMALE		
Controls	XIV–XIX	0.54 $\pm$ 0.028	0.55 $\pm$ 0.071	50.4 $\pm$ 2.48
Insulin injected	XX	0.63	0.55	53.4
Insulin injected	XXI	0.27	0.51	34.6
Insulin injected	XXII	0.23	0.50	31.5

mal magnitude. In these animals pigment was formed in the suprarenal medulla after oxidation with potasssium iodate in the same way as in the medulla of the control cat. After oxidation with potassium bichromate, however, 60–70% of the cell complexes demonstrated marked reduction of their pigment formation potential. In two cats (No. XII and XIII, Table 1) insulin injection caused an even more pronounced adrenaline depletion, yet the noradrenaline content remained essentially unaltered. Following oxidation with potassium iodate the cytological picture of the suprarenal medulla was still normal: 30–40% of the cell complexes displayed pigment formation of usual intensity and localized within well delineated areas (Fig. 1c). After oxidation with potassium bichromate on the other hand the

cytological picture differed most markedly from the normal: 60–70% of the cell complexes produced no pigment formation at all or a very slight one, while the remaining 30–40% of the complexes still give intense pigment formation (Fig. 1d).

In the rat insulin induced hypoglycemia produced changes in the cytological picture of the suprarenal medulla analogous to those found in the cat. Thus increased secretory activity on oxidation with potassium bichromate gave rise to a reduced pigment formation. This reduced capacity to pigment formation was proportional to the length of time and intensity of the stimulation; at maximal stimulation pigment formation was absent in all but 5–15% of the medullary cells. Following oxidation with potassium iodate the adrenal medulla in *all* animals treated with insulin presented pigment formation in 5–15% of the cells, which is the same as in the medulla of the nontreated rat. In the rat experiments quantitative determination of adrenal catechol amines was not performed. Thus the cytological picture could not be correlated directly to the catechol content after stimulation. In identical experiments, where such determinations were carried out, prolonged hypoglycemia was found to be followed by total depletion of the adrenaline content without any appreciable decrease of the noradrenaline content (Hökfelt, 1951).

DISCUSSION

If those medullary cells which after oxidation with potassium iodate give pigment formation represent only functional variations of cell activity one would expect a change in the number of the pigment forming cells as well as a change in the intensity of the pigment reaction as a result of the varied secretory activity in the suprarenal medulla produced by denervation or insulin induced hypoglycemia. However, after greatly reduced secretion or markedly increased secretion of adrenaline no changes of the reactivity of the suprarenal medulla to potassium iodate could be seen. These experiments thus give further strong support to the *specificity* of those cells which after oxidation with potassium iodate show pigment formation.

After depletion of the adrenaline content of the suprarenal medulla but without any appreciable decrease of the noradrenaline content, those cells which under normal conditions present pigment formation after oxidation with potassium iodate remain unchanged. Those cells on the other hand, which normally give rise to pigment formation only when oxidized with potassium bichromate gradually lose this capacity, in proportion to the gradual adrenaline depletion. In all probability the potassium iodate reaction introduced by us directly represents noradrenaline within the cells. Thus further evidence has been obtained to show that the specific cells

demonstrated, are specifically concerned with the storage of noradrenaline.

The cytological findings described in this paper are in agreement with the physiological view that noradrenaline and adrenaline are two specific hormones which are selectively secreted from the adrenal medulla under different conditions. This hypothesis was first presented by Euler (1950) and experimental data are accumulating supporting this view. Thus insulin induced hypoglycemia causes a selective depletion of the adrenaline content of the suprarenal gland in the rat and the cat (Hökfelt, 1951, 1953), while a marked diminution, especially of the adrenaline secretion from the suprarenal medulla is bought about by elevation of the blood glucose level (Dunér, 1953). Furthermore electrical stimulation within different hypothalamic areas have been shown to cause a selective output of noradrenaline or adrenaline in the suprarenal venous blood (Brücke, Kaindal and Mayer, 1952, Euler and Folkow, 1953).

SUMMARY

1. Oxidation of the suprarenal medulla with potassium iodate produces a specific pigment formation of noradrenaline.

2. Using this method experimental evidence has been put forward to show the existence of specific "noradrenaline cell complexes" within the suprarenal medulla. This is strongly supported by the fact that in the cat and in the rat insulin induced hypoglycemia, which causes a gradual depletion of the adrenaline content of the suprarenal gland without any decrease of the noradrenaline content, does not alter either the number of the noradrenaline pigment forming cells or the intensity of the pigment reaction.

Acknowledgments

We want to express our gratitude to Professor U. S. v. Euler for his interest and helpfulness in all our investigations concerning the suprarenal medulla.—The investigations have been supported by grants from Statens Medicinska Forskningsråd and Stiftelsen Therese och Johan Anderssons Minne.

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ALKALINE PHOSPHATASE, GLYCOGEN, AND PERIODIC ACID-SCHIFF POSITIVE SUBSTANCES IN THE BOVINE UTERUS DURING THE ESTROUS CYCLE

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INTRODUCTION

THE general histology of the uterus of the cow was described by Schmaltz (1911) and histological changes during the course of the estrous cycle were reported by Murphey (1924), Hammond (1927), Cole (1930), Asdell (1946), Asdell *et al.* (1949), and Weber *et al.* (1948 and 1950). Thus far no histochemical findings have been reported on the bovine uterus, although numerous studies have been made on those of other species. The general distribution of alkaline phosphatase in the uterus of various species has been reported by Gomori (1941), Kabat and Furth (1941), and Wislocki and Dempsey (1945). Changes in alkaline phosphatase distribution during the estrous cycle were given for the rat by Pritchard (1949), for the human endometrium by Atkinson and Engle (1947) and Arzac and Blanchet (1948). Cyclic variation of the uteri of several species with regard to glycogen distribution has been demonstrated by many investigators (Reynolds, 1949). Among recent investigators are Spyker and Fidler (1942), Zondek and Shapiro (1942), Hughes (1945), Boettiger (1946), Arzac and Blanchet (1948), and Bo and Atkinson (1952). These studies indicate a relationship of alkaline phosphatase and glycogen to reproductive processes. The present invstigation was undertaken as part of our general studies of the bovine reproductive tract in connection with studies on bovine infertility.

MATERIALS AND METHODS

Tissues for these studies were obtained from 20 animals which were killed at known stages of the estrus cycle. Their ages ranged from 15 to 110 months. These animals appeared to be normal as judged by regularity of cycle length. All had shown at least 4 regular cycles prior to slaughter. One or more animals were killed at days 0, 1, 2, 3, 5, 8, 9, 10, 12, 13, 14, 16, 18, 19, and 21 of the cycle. Tissue specimens were collected immediately after slaughter, fixed in cold acetone, absolute alcohol, and Carnoy's fluid, dehydrated as usual and infiltrated in vacuo in low melting point paraffin.

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Alkaline phosphatase was determined by the modified cobalt sulfide method of Gomori (1952). Sections fixed in acetone and absolute alcohol were cut at 8 μ and incubated in a solution containing Na-glycerophosphate, buffered with veronal to pH 9.3 for 1, 2, 4, and 16 hours at 37° C. Some sections were also treated with the diazo method of Menten *et al.* (1944), and that of Manheimer and Seligman (1948). Control sections were incubated in a solution from which either the substrate had been omitted or in the same solution after treatment with trichloroacetic acid. Glycogen and periodic acid-Schiff (PAS) positive substances were determined on tissues fixed in Carnoy's fluid and some also fixed in absolute alcohol. Sections were cut at 5 μ and treated with the PAS technique of McManus (1946), and Hotchkiss (1948). The Schiff reagent was prepared according to the modification of Lillie (1951). Control sections were incubated in a dilute saliva solution prior to staining. Some sections were also treated with the Best's carmine or the Bauer-Schiff procedure for glycogen (Lillie, 1948).

RESULTS

Alkaline Phosphatase

A comparison of the diazo methods with the cobalt-sulfide method indicated that the distribution of phosphatase was essentially the same irrespective of the method used. In the description of phosphatase distribution that follows, unless stated otherwise, the observations are based on acetone fixed tissues using the cobalt-sulfide method and a 4 hour incubation period. Comparison of tissues incubated for 2, 4, and 16 hours indicated that the 4-hour incubation period was most suitable generally for the demonstration of alkaline phosphatase in the structures of the bovine uterus.

In the endometrial stroma, phosphatase is always present in very large quantities in intercotyledonary areas in the upper densely cellular zone. In cotyledonary areas the enzyme is present in this zone only in capillary endothelium. In the middle and lower zones of the endometrium phosphatase is also always present in very large quantities in the fibrous sheaths of the gland tubules and blood vessels and in the areolar connective tissue. Figures 1 and 2 show the distribution of phosphatase in these areas. The amount of phosphatase activity present at these locations directly parallels the degree of fibrous development. The overall phosphatase of the areolar tissue is always considerably less than that of the fibrous sheaths surrounding the glands. In the corpus uteri where fibers are well developed at the base of the endometrium phosphatase activity is proportional to the degree of fibrous development. The extent of fibrous development and hence the amount of phosphatase present in all these locations decreases from the tip of the horn towards the corpus uteri. There is also an apparent increase of fibrous development and phosphatase activity throughout the uterine horn at the middle of the cycle.

At the base of the endometrium fibrous trabeculae often containing gland tubules are frequently seen penetrating the circular muscle at right angles (Fig. 3). As these trabeculae pass through the circular muscle layer,

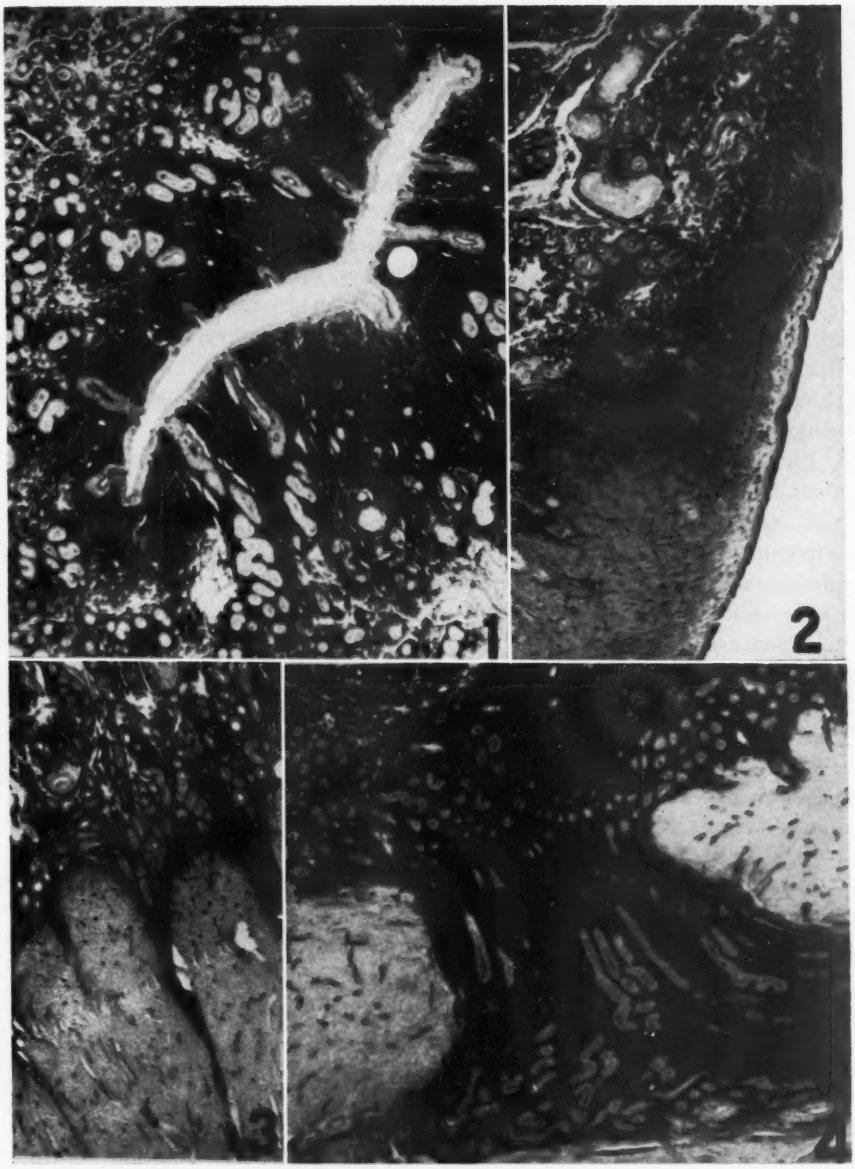


FIG. 1. Alkaline phosphatase of the uterine horn 5 days after beginning of estrus. Note high activity in the upper endometrium, lack of activity in the surface epithelium, and gradation of activity of endometrial glands with depth. ×41.

FIG. 2. Alkaline phosphatase activity of upper endometrium. Note high activity in the intercotyledonous upper endometrial stroma and the lack of activity in the cotyledonous area. ×41.

FIG. 3. Alkaline phosphatase activity of basal zone of endometrium. Note high activity of the fibrous trabeculae and absence of activity in the myometrium. ×41.

FIG. 4. Alkaline phosphatase activity of endometrial protruberance invading the myometrium. Note high activity of the fibers at the constricted portion. ×41.

the fibers become compressed with no areolar connective tissue between them. The gland tubules become narrow, straightened, and are close to each other. Towards the periphery of the circular muscle the fibrous bundles separate again and branch out from each other giving rise to loose areolar connective tissue between them. Also the gland tubules become coiled again and widen resulting in the entire structure having the same appearance as the intermediate zone of the endometrium (Fig. 4). These structures have high phosphatase activity which also increases with increase in fiber density. Thus, at the neck of the protuberance, where the fiber density is highest, the phosphatase activity is also highest, but as the fibers thin out in the periphery of the circular muscle the phosphatase activity also diminishes accordingly.

Phosphatase activity is also present in the endometrium in the endothelium of capillaries and small blood vessels, but not in those of medium and large blood vessels. Large blood vessels contain the enzyme in the outermost coat of the adventitia. No cyclic variation with regard to phosphatase distribution in blood vessels could be detected.

The distal border of the cells of the surface epithelium always contains phosphatase. Aside from it, there is, however, a marked variation in the phosphatase of the epithelial cells during the estrous cycle. The variation in phosphatase is more pronounced in this location than in any other structure in the uterus. From Table 1 it can be seen that for a few days before and for at least 5 days after the onset of estrus, phosphatase activity is very low in the surface epithelium. From at least day 8 to day 17 there is a marked increase in phosphatase activity. The greatest concentration occurred about days 10–12. This variation is illustrated in Figures 5 and 6.

The phosphatase activity of the glandular epithelium shows a gradation with depth, activity being highest in the superficial glands and diminishing to low or no activity near the base of the endometrium. The cells of the superficial glands always contain high phosphatase activity along the distal border and phosphatase activity is also seen in the secretion, when present, in the wide portion of the lumen (Figs. 1, 5, and 6). The cytoplasm of the superficial gland cells always contains some phosphatase, but no consistent variation in cytoplasmic phosphatase similar to that seen in the cells of the surface epithelium was observed during the cycle as seen in Table 1. Cells of glands of intermediate depth usually have a thin darkly staining distal border and faintly staining nuclei. Towards mid-cycle considerable activity might also be present in the cytoplasm of the glandular cells but there is much more individual variation in phosphatase content here than in the more superficial glands. The cells of the basal (deepest) glands often have a fine darkly staining distal border and faintly staining cell nuclei

TABLE 1. VARIATION OF UTERINE ALKALINE PHOSPHATASE, GLYCOGEN, AND PAS POSITIVE CELLS DURING THE COURSE OF THE ESTROUS CYCLE

With regard to gradation of amounts in this table, it is assumed that each particular category has different criteria. Thus, the number of PAS cells found to be maximum (and marked +++++) in one particular section is still very low in number when compared with the number of glycogen granules found in traces only (and marked ±)

Days after estrus	Site	Surface epithelium		Superficial glands		Glycogen of		PAS positive cells
		P	G	P	G	Cotyledonous upper endomet. stroma	Longit. muscle	
0	UH	++	+++	++	+	−	+++	+
	U	++	++	++	++	−	+	+
0	UH	+	+++	+	+++	−	+++	+
	U	+	++	+	++	−	−	+
1	UH	+	+++	++	+	−	+++	−
	U	±	++++	+	+	−	−	++
1	UH	+	+++	++	+	+	+++	±
	U	±	++	++	+	−	−	±
2	UH	+	+++	+	++	−	±	−
	U	±	++++	++	+++	−	−	−
3	UH	+	++++	+	++++	±	−	−
	U	+	+++*	+	+++	−	−	−
3	UH	+	++++	+	++	−	+++	−
	U	+	++++	++	++	−	++	−
5	UH	+	++++	+	+++	−	+++	−
	U	±	++++	+	++++	+	++	−
8	UH	+++	−	++	+++	±	+++	+
	U	+++	−	++	++	+	+++	+
9	UH	+++	−	±	++	±	+++	+
	U	++++	−	±	−	+++	++	++
10	UH	++++	−	+	±	−	++	+
	U	++++	−	++	+	++++	++	−
12	UH	++++	−	++	+++	−	+++	+
	U	+++	−	+	+++	+++	+	++++
12	UH	+++	−	++	++	+	++	±
	U	+++	−	++	++	++	++	±
13	UH	+++	−	+	+	±	+++	+
	U	++++	−	++	++	++++	++++	++++
14	UH	+++	+	++	+++	+	++++	−
	U	++	++†	+	+	−	+	+++
16	UH	+++‡	++++	++	++	−	+	+
	U	+++	++	+++	++	−	−	+
17	UH	+++	+++	+	++	±	+++	±
	U	+	+++	+	++	−	−	++
18	UH	++	+	++	+++	not det.	++++	++
	U	++	+++	++	+	−	++‡	++
19	UH	+	++++	++	++	−	+++	−
21	U‡	++	++++	++	+++	−	+	+++
	UH	+	++++	+	++	−	−	++
	U	++	++++	++	+++	−	−	+++

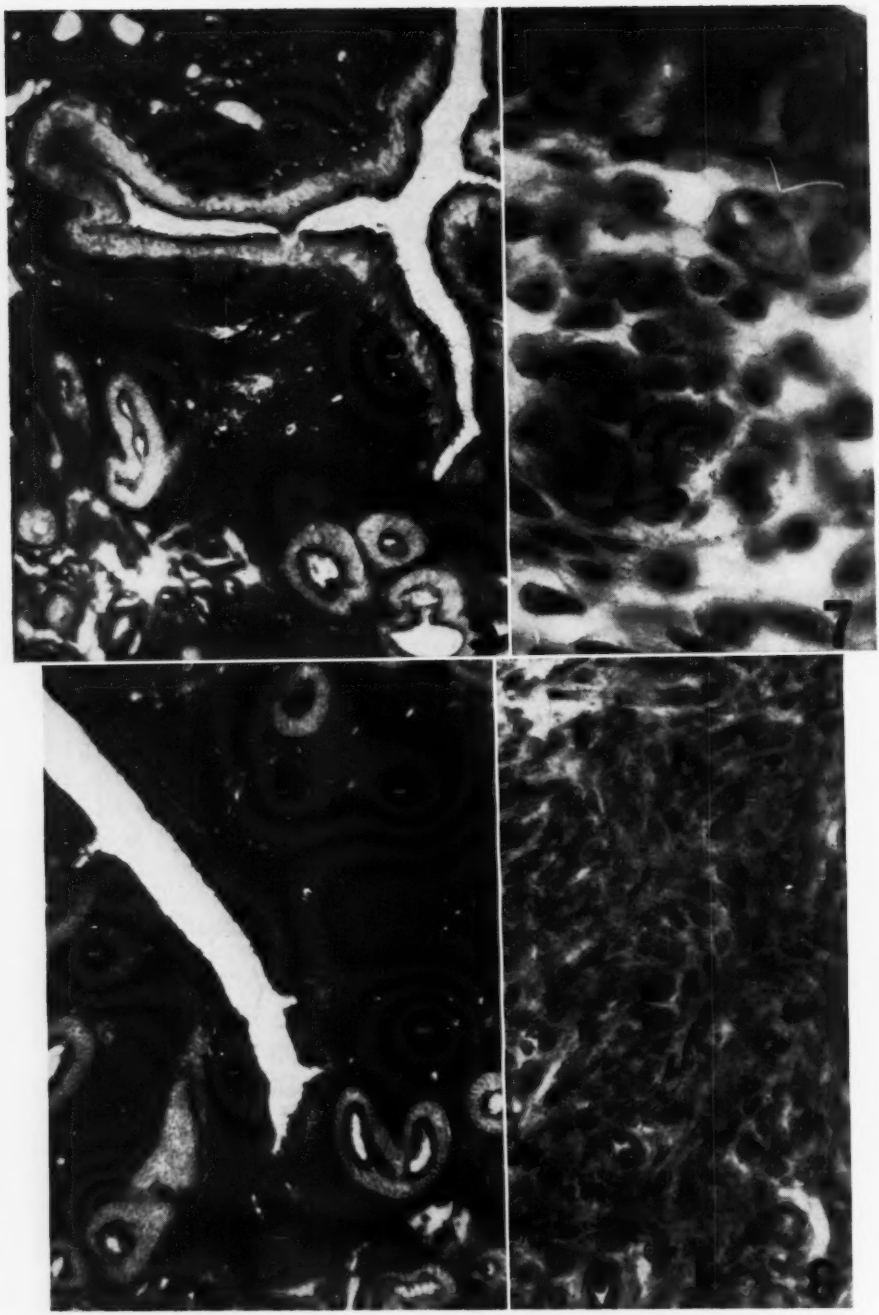
P = alkaline phosphatase.
G = glycogen.
UH = section near tip of uterine horn.
U = section near corpus uteri.
not det. = tissue section not analyzed.
− absent.
± trace.
+ small amount.

* Best method.
† Bauer and Best methods.
‡ Absolute alcohol fixed tissue.

++ moderate amount.
+++ large amount.
++++ very large amount.

about mid-cycle. At other times basal glands do not stain after a 4-hour incubation period although a faint stain occasionally developed after 16 hours incubation.

The myometrium contains phosphatase activity in the capillary endothelium and occasionally after prolonged incubation also in the longitudinal muscle. The circular muscle never contains any trace of phosphatase activity even after 16 hours incubation. No phosphatase activity was seen in any part of the epimetrium, aside from capillary endothelium.



FIGS. 5–8

Glycogen and PAS Positive Substances

We found that the PAS method consistently demonstrated larger concentrations of glycogen than either the Bauer or Best methods. There are no detectable differences in the amount of glycogen present in the tissues after Carnoy fixation from that of absolute alcohol fixed tissues. Our findings as given below, are based on Carnoy fixed tissue treated with the PAS technique unless stated otherwise. Glycogen granules, when present, are always dispersed in the cell cytoplasm, and were never seen in cell nuclei.

In the endometrial stroma glycogen granules are present in small round cells which are more concentrated in the upper endometrium beneath the surface epithelium (Fig. 7) and are seen more often in the corpus uteri than in the horn. The variation in the distribution of these cells could not be related to the cycle. Loose glycogen granules are often seen in abundance in the cotyledonous upper endometrial connective tissue stroma of the corpus uteri (Fig. 8) and seem to be more concentrated during the midcycle as seen from Table 1.

The greatest change in glycogen distribution during the estous cycle was seen in the surface epithelium. As seen from Table 1 and Figures 9, 10, and 11, glycogen is present in the surface epithelium in very large quantities for the last few days before (Fig. 9) and for at least the first 5 days after (Fig. 10) the beginning of estrus. From at least day 8 to day 13, at the time of very high phosphatase activity, no glycogen at all is seen in the surface epithelium (Fig. 11). Although some irregularity in the distribution of glycogen in some areas of the surface epithelium was seen there was no consistent difference in glycogen distribution between cotyledonary and intercotyledonary areas, nor in height of epithelium, degree of stratification or between different locations in the same uterus.

With regard to the superficial glands it was noted that at least some of these always contain some glycogen in their gland cells as well as in their gland lumina. Frequently large amounts of glycogen are seen in some of the superficial gland cells and lumina even when no glycogen at all is present in the surface epithelium. Furthermore, even when most superficial glands

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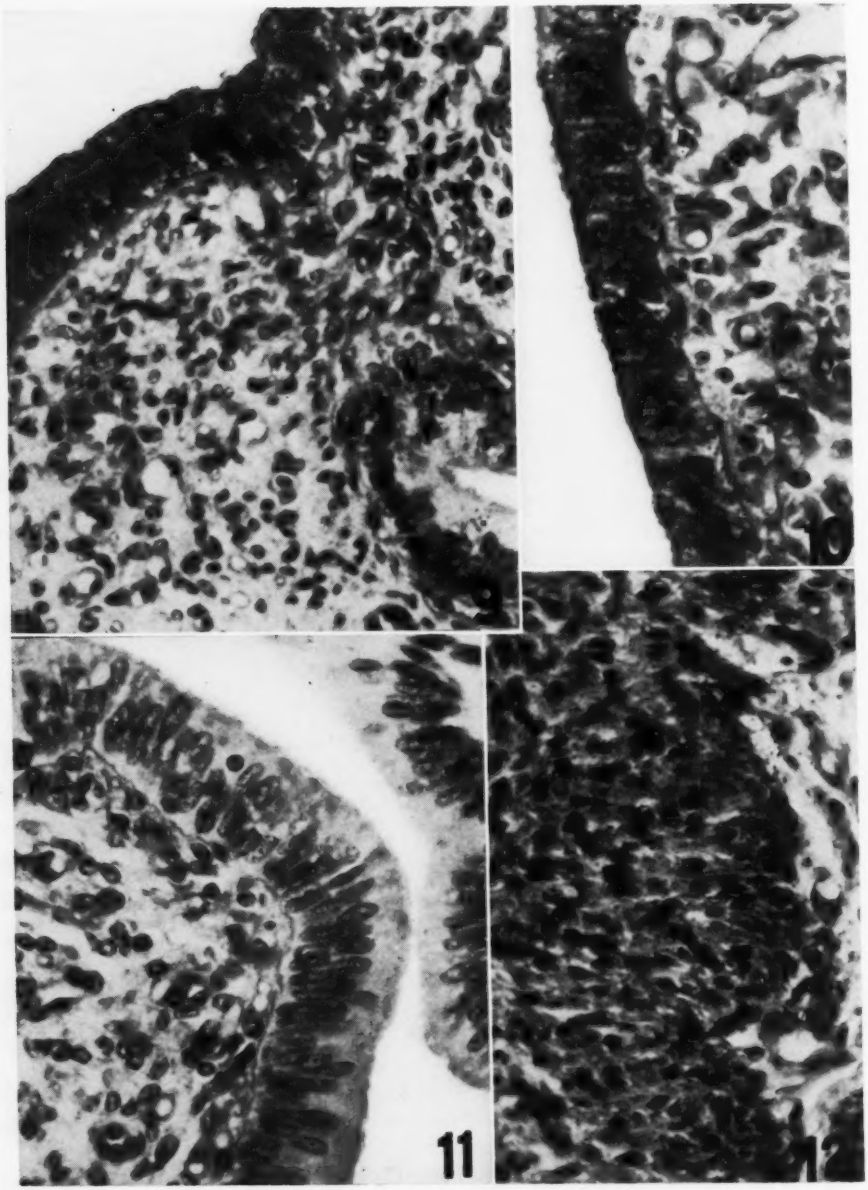
FIG. 5. Uterine horn at estrus. Alkaline phosphatase activity. Note absence of activity in the surface epithelial cells, except at their distal borders. ×113.

FIG. 6. Uterine horn 9 days after beginning of estrus. Alkaline phosphatase activity. Note high activity of surface epithelium. ×113.

FIG. 7. Group of glycogen containing cells in the upper endometrium. PAS technique, photographed with Wratten filters nos. 15 and 58. ×1045.

FIG. 8. Glycogen and PAS positive cells (upper center) in the upper endometrial stroma. PAS technique, photographed with Wratten filters nos. 15 and 58. ×500.



FIGS. 9–12

contain maximum amounts of glycogen there are usually some seen with no glycogen in either cells or lumina. From Table 1 it is seen that no correlation could be established between the amount of glycogen in the superficial glands and the stage in the estrous cycle. Glycogen granules are seldom present in intermediate and never in basal glands.

In the myometrium glycogen is present in very large quantities in the walls of many blood vessels in the stratum vaculosum. In addition large quantities of glycogen are present in parts of the longitudinal muscle (Fig. 12), the distribution of which may possibly be related to the stage in the cycle, as seen from Table 1. The circular muscle layer adjacent to the endometrium never contains glycogen, although traces of glycogen may occasionally be found in the circular muscle around the stratum vasculosum. No glycogen is ever seen in any part of the epimetrium.

At certain stages in the cycle, shown in Table 1, the upper endometrial stroma contains typical small cells, with peripheral small round nuclei, the cytoplasm of which stains brilliantly red with the PAS technique and give the typical stain with the Bauer and Best methods for glycogen. These cells are illustrated in Figure 8. The substance being stained is not glycogen since the stain is present even if sections are treated with saliva prior to staining. Occasionally larger patches of this stain are seen where the cellular outline of individual cells is lost. This probably resulted from the coalescence of 2 or more cells containing PAS positive material. These structures are similar in coloration to the PAS positive colloid which was found in some granulosa lutein cells of the ovarian corpus luteum. These cells are more numerous in areas near the corpus uteri than in areas near the tip of the horn. Furthermore, they ae found in larger concentration in cotyledonous than in intercotyledonous areas. Occassionally some cells are seen in deeper zones of the endometrial stroma. It is possible that these cells represent some stage of activity of the glycogen containing cells which were described above.

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FIG. 9. Uterine horn 16 days after beginning of estrus. PAS technique, photographed with Wratten filters nos. 15 and 58. Note high concentration of glycogen in the surface epithelium. ×500.

FIG. 10. Uterine horn 1 day after beginning of estrus. PAS technique, photographed with Wratten filters nos. 15 and 58. Note high concentration of glycogen in the surface epithelium. ×500.

FIG. 11. Uterine horn 10 days after beginning of estrus. PAS technique, photographed with Wratten filters nos. 15 and 58. Note absence of glycogen from the surface epithelium.

FIG. 12. Glycogen in the longitudinal muscle of the uterus. PAS technique, photographed with Wratten filters nos. 15 and 58. ×500.

Since Schmaltz (1911) has stated that the surface epithelium always lacks a basement membrane it is of interest to show a very distinct basement membrane of the surface epithelium which is demonstrated very frequently with the PAS technique (Figs. 7, 9, 10, 11). The gland cells of the tubules, however, do not show such a basement membrane.

DISCUSSION

The cow uterus seems to differ significantly from that of other species in that large amounts of alkaline phosphatase are found in the endometrial connective tissue stroma. Little activity in the endometrial stroma was seen for the human by Atkinson and Engle (1947) and by Arzac and Blanchet (1948) and for the rat by Pritchard (1949). In the cow the activity of the fibrous endometrial connective tissue is always by far greater than that of any of the epithelial cells aside from the distal border of the surface epithelium and the superficial gland cells. Furthermore, the amount of phosphatase in the endometrial stroma increases directly with increase in density of the stroma cells and fibers, and shows cyclic variation only to the extent of fiber density variation within the cycle.

The connective tissue fibers of the uterus show phosphatase activity only in those areas and structures which also contain glands. Thus the fibers of the glandular sheaths, which are continuous with the fibers of the stratum vasculosum contain phosphatase activity whereas the fibers of the stratum vasculosum are inactive. Furthermore, the fibrous trabeculae which penetrate the circular muscle and which do not carry glands across the circular muscle gradually lose phosphatase activity as their association with the glands is lost. Trabeculae with glands always have phosphatase activity. The phosphatase activity of these fibers appears to depend on an association with glandular structures.

Pronounced and distinct cyclic variation of phosphatase activity of the bovine uterus is present only in the surface epithelium. Cyclic variations in human endometria have been observed by Atkinson and Engle (1947) and Arzac and Blanchet (1948). There the greatest amount of phosphatase in the surface epithelium was found to occur during the estrogenic phase of the cycle with a decrease occurring after ovulation during the progestational phase of the cycle. In the bovine, we find less phosphatase in the surface epithelium prior to and following ovulation than at mid-cycle. In this respect phosphatase distribution in the bovine eipthelium more closely resembles the rat than the human although some difference appears to exist between these species also. Pritchard (1949) has observed that in the rat the distal band of the surface epithelium possesses the highest activity in proestrus and early estrus and a minimum of activity in diestrus, but the phosphatase activity of the surface cell nuclei and distal cytoplasm (aside

from the distal band) is lowest during proestrus and highest during diestrus. Our observations in the bovine indicate that the phosphatase of the distal band is always very high and does not show definite fluctuations during the cycle. However, variation in phosphatase activity in other parts of the cell are similar to those for the rat, being lowest during estrus and highest during diestrus.

It is generally agreed that the amount of glycogen in the human endometrium is maximal during the progestational phase of the cycle, absent during menstruation and minimal during the estrogenic phase of the cycle (Reynolds, 1949). Arzac and Blanchet (1949) have shown that glycogen is increased in both epithelium and the endometrial stroma during the progestational phase of the cycle and is absent during menstruation. In the bovine we have found little or no glycogen in the endometrial stroma except for the upper zone in cotyledonary areas where lots of glycogen is often seen in the corpus uteri where the cotyledons are well developed. The surface epithelium contains glycogen prior to and following ovulation but none during mid-cycle. Both the human and the bovine differ from the rat in that the rat endometrium (Bo and Atkinson 1952) does not contain glycogen. The distribution of glycogen in the bovine differs from the rat in one further respect. Bo and Atkinson (1952) have shown that glycogen in the rat is mainly confined to the longitudinal muscle and that it is maximal during proestrus and estrus at which time the circular muscle may also irregularly show glycogen deposition. Glycogen has never been observed in our series in circular muscle and glycogen appears to be maximal in the longitudinal muscle during diestrus.

Our observations seem to indicate that there is an inverse relationship between the amounts of alkaline phosphatase and glycogen in the several locations where these substances are found in the bovine uterus. During mid-cycle, when a marked increase in phosphatase occurs in the surface epithelium, glycogen is absent; little or no glycogen is seen in the endometrial stroma which is always highly phosphatase-active, the upper endometrial stroma in cotyledonar areas which lacks phosphatase (aside from capillary endothelia) often shows considerable glycogen, and glycogen occurs regularly in the longitudinal muscle which, except for capillaries, is negative for phosphatase (unless the incubation period is unduly prolonged). Alkaline phosphatase and glycogen may be present together but where this situation exists the concentration of phosphatase is relatively low. From Table 1 it can be seen that the concentration of phosphatase in the epithelium of the superficial glands is relatively low throughout the cycle and glycogen also is present throughout; when the amount of phosphatase in the surface epithelium is relatively low and comparable to that of the superficial glands, glycogen is present in amounts similar to that seen in

the superficial glands. These observations could be interpreted to mean that alkaline phosphatase activity is necessary for glycogen utilization and that accumulation of glycogen occurs because phosphatase activity is low or absent. A similar inverse relationship to the above can be observed in the human endometrium where phosphatase activity is high during the estrogenic phase of the cycle and low during the progestational phase at which time glycogen is increased.

SUMMARY

Alkaline phosphatase and glycogen were determined on tissue sections obtained from 20 cows during various stages of the estrous cycle.

In the endometrium alkaline phosphatase was always present in the intercotyledonary areas of the upper, densely cellular stroma, in the fibrous sheaths of gland tubules and blood vessels, and in the distal borders of the surface epithelial and superficial gland cells. The capillary endothelium showed phosphatase activity in all parts of the uterus.

A distinct cyclic variation of phosphatase activity was seen only in the surface epithelial cells (aside from their distal borders). In these the highest activity was seen during the mid-cycle, but little or no activity was present for several days at the beginning and at the end of the cycle.

The presence of phosphatase activity in the stroma fibers of the uterus seems to be dependent on an association with the endometrial glands. The amount of phosphatase activity present in the endometrial stroma seems to be related to the extent of fibrous development.

Glycogen was often found inside small round cells in all parts of the upper endometrial stroma or loose in the cotyledonous, upper endometrial stroma, in superficial and occasionally also in intermediate glands, in the walls of some large blood vessels of the myometrium, in the longitudinal muscle and in the surface epithelium.

A pronounced cyclic variation of glycogen distribution was found only in the surface epithelium. However, in contrast to phosphatase distribution the largest amount of glycogen was seen for several days at the beginning and at the end of the cycle, but no glycogen was found during the mid-cycle.

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THE RESPONSE OF HYPOPHYSECTOMIZED MALE KILLIFISH TO PURIFIED FISH GROWTH HORMONE, AS COMPARED WITH THE RESPONSE TO PURIFIED BEEF GROWTH HORMONE[1]

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IN PREVIOUS experiments (Pickford and Thompson, 1948; Pickford, 1953b) it was shown that both normal and hypophysectomized killifish (Fundulus heteroclitus L.) respond to injections of purified beef growth hormone. In both series of experiments two phenomena were observed: (1) There was a period of several weeks before growth commenced, and (2) During this period both the control fish and those receiving growth hormone showed a progressive loss of weight. This trend was reversed at the end of 3 to 4 weeks in fish receiving growth hormone. The initial period of weight loss was attributed in part to the disturbances resulting from handling but, in the case of fish receiving growth hormone, it was suspected that the recipients reacted adversely to a foreign protein of mammalian origin.

The problem of dosage level had also to be considered. The beef growth hormone preparations were highly purified samples capable of inducing a weight increase of 10 gm. in 10 days at a dosage level of 10 μg./day when injected into hypophysectomized rats. Assuming the weight of the rats to be 100 gm. this is equivalent to 0.1 μg./gm. weight/injection. However, the first experiments showed that fish are sensitive to frequent handling and it was found advisable to make the injections on alternate days, thrice weekly. At a dosage level equivalent to that which induces growth in rats the fish do not respond at all. The minimal dose at which a weak response could be obtained in normal fish was about ten times greater than the minimal dose required by rats, 2μg./gm. weight thrice weekly, equivalent to 0.85 μg./gm. weight on a daily injection schedule. A more adequate response was obtained at a higher dosage level, 10 μg./gm. weight thrice weekly, which is fifty times greater than that required by rats. Several reasons might be advanced to explain this low order of sensitivity in fish. In the first place it might be a temperature effect. In the second place it might be due to an unfavorable response of the fish to donor material of mammalian

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origin. The experiments on hypophysectomized fish, which respond in the same manner as normal fish, eliminated the possibility that the lower response could be due to a lesser sensitivity in normal animals.

The effect of temperature on the response of fish to growth hormone preparations is now under investigation and the results will be reported in a later communication. In order to examine the second hypothesis it was necessary to study the activity of growth hormone preparations derived from fish pituitaries[2] and the present report is concerned with this investigation.

MATERIALS AND METHODS

Collection of the glands. In the summer of 1950 about 325 gm. of fresh frozen fish pituitaries were collected from an estimated 16,700 heads; about 25 gm. were from hake, Urophysis tenuis (Mitchill), and the rest mostly from pollack, Pollachius virens (Linn.), with a small admixture of cod, Gadus morhua Linn. From this material Dr. Wilhelmi prepared the first sample of purified fish growth hormone (F6B). In the summer of 1952 a second batch of about 400 gm. was collected, mostly from hake admixed with about 50 gm. from pollack and cod; a total of about 34,000 heads were used since the hake gland is smaller than that of the pollack. From this material Dr. Wilhelmi prepared a second, more highly purified sample of fish growth hormone (F8OGH).

The procedure for collecting the glands may be briefly described. Freshly caught fish when brought to the wharf are hoisted, weighed and dressed. The heads, which are removed first, were taken by a man whom we employed to split them. After trying several methods with cleavers and hatchets we found that it is most satisfactory to use a saw. The head is placed on a wooden block provided with a cleat to hold it in place and is cut open transversely about half an inch behind the eyes. The exact position of the cut depends on the species and on the size of the head and must be determined by experience. The cut should extend half way through the head which may then be opened and the gland, conveniently exposed in the posterior half when the optic nerves are lifted, can be picked up with a pair of fine forceps. The glands were collected in 1 ml. beakers suspended by means of a wire frame in a bowl of ice water. As each beaker was filled excess fluid was drawn off with a fine medicine dropper and the glands were transferred to a paraffin-lined can in the deep freezer. About 50 pollack pituitaries are required to fill a 1 ml. beaker wheras 70–80 hake glands are necessary. One man cutting open the heads can provide enough material for two gland collectors; we found that about 200 glands could be collected in this way in an hour. The number of fish brought in at one time varied from a few hundred or less to several thousand; they might have been caught by hook and line, seined or taken from a weir but they were always in good condition, generally only a few hours old. The flesh was firm and frequently the hearts were still beating. After several hours of work we noticed that the flesh began to soften and slipped readily from the skull. Collection of glands was discontinued when the material was no longer in good condition but, as working temperatures were usually low, deterioration was not rapid. On one or two occasions the fish remained overnight, protected by chopped ice, and were still in good condition in the morning. While it must be assumed that ma-

[2] I am indebted to Dr. A. E. Wilhelmi of the Department of Biochemistry, Emory University, who prepared the samples of purified fish growth hormone and whose studies of this material are about to be reported in a separate communication.

terial so collected might show some loss of hormonal activity every effort was made to avoid deterioration. The frozen glands were shipped to Dr. Wilhelmi packed in dry ice.

Experimental procedures. The experiments were made in the same manner as in the two previous investigations but one or two improvements were introduced. Installation of a cooling unit[3] permitted the work to be done at a constant temperature (20° C.). The aquarium room was kept cold and individual heaters with thermostatic controls were installed in the tanks. As before, the tanks were illuminated for 8 hours per day. The fish were fed daily with iodinated liver-pablum; on injection days they were fed in the morning and injected in the afternoon since fish that have been recently disturbed will not eat. A dietary supplement of frozen raw daphnia was given two or three times weekly. This food supplement and the constant warm temperature of the aquaria represent two major improvements of procedure that may have contributed to the more prompt and vigorous response to the growth hormone injections, as compared with previous experiments. In addition the fish were given a 30 second dip in 1:15,000 zinc-free malachite green at weekly intervals, against fungus infections, and the tanks were supplied with more vigorous aeration.

A trial experiment, which will not be reported in detail, was made with the first fish growth hormone preparation (F6B). The response was of a lower order than that elicited previously with purified beef growth hormone (Table 1). At a dosage level of 10 μg./gm. weight 4 fish showed an average weight increase of 8.4% after 8 weeks, and an average length increase of 2.1%. No response was observed at the 2 μg. dosage level. In contrast to previous experiments the fish showed no initial period of weight loss preceding the resumption of growth. It was thought that this more favorable reaction might be due to the use of fish rather than beef growth hormone but the improved experimental conditions introduced a second factor. In the next experiment, the subject of the present communication, it was decided to make a simultaneous comparison of the two preparations.

For the definitive experiment two-year old male fish were hypophysectomized in the manner described previously (Pickford, 1953a) between Feb. 16 and Feb. 20, 1953, and allowed to come to physiological equilibrium. Six weeks later, at the start of the experiment, the nuptial colors had disappeared and no fish showed growth in length since the date of operation. Beginning on March 30 the fish were injected intraperitoneally thrice weekly at a dosage level of 10 μg./gm. weight. One group of 7 fish received fish growth hormone (F8OGH). A second group of 7 fish received beef growth hormone. For this purpose No. 568 (Wilhelmi) was used during the first two weeks (6 injections) but was discontinued because of the development of nuptial coloration. Five injections were made with preparation No. 508B (Wilhelmi), known from a previous experiment to be free from this collateral activity, and when this material was exhausted the experiment was completed with Organon No. 66450.[4]

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[3] Thanks to a grant from the Higgins Foundation which is gratefully acknowledged.

[4] The following information is available regarding the growth promoting activities of these preparations when tested on rats. According to Wilhelmi (1954) "The fish preparations have been tested in the conventional growth assay test in hypophysectomized male rats. Daily injections of 0.02, 0.2, or 2 mg. per day for 10 days led to uniformly the same result: there was a small gain in body weight for the first 4 days, followed by a loss in weight for the remainder of the period, so that most of the test animals weighed two to four grams less at the end of the test than they did at the beginning." I am indebted to Dr. Wilhelmi (in litt.) for the following information regarding the beef growth hor-

TABLE 1. SUMMARY OF THE RESULTS OF A PREVIOUS EXPERIMENT IN WHICH THE RESPONSE OF HYPOPHYSECTOMIZED MALE KILLIFISH TREATED WITH GROWTH HORMONE AT 10 μG. PER GRAM WEIGHT THRICE WEEKLY WAS COMPARED WITH THAT OF UNOPERATED, MOCK OPERATED, UNTREATED HYPOPHYSECTOMIZED AND SALINE INJECTED HYPOPHYSECTOMIZED CONTROLS[1]

Treatment	N	Mean or standard deviation	Length change %	Weight change %	Scales, no. new circuli	GSI[2]	HSI[3]	TEH[4] μ
Unoperated[5]	7	M	—	—	—	4.00	3.2	9.5[6]
		s	—	—	—	1.15	0.6	3.6
Mock operated	6	M	6.4	12.3	8	5.60	2.8	12.5[6]
		s	2.0	8.5	—	0.81	0.5	2.5
Hypophysectomized, untreated	6	M	0.8	−10.0	0	0.41	6.6	3.6
		s	1.1	7.6	—	0.18	2.8	0.8
Hypophysectomized, saline injected[7]	4	M	0.0	−17.1	0	0.23	5.1	3.8
		s	0.5	3.1	—	0.14	0.9	0.8
Hypophysectomized, beef growth hormone[7]	7	M	4.2	14.8	3.5	0.49	4.6	5.2
		s	1.4	11.1	—	0.16	0.6	0.6

[1] Pickford (1953b).
[2] Gonosomatic index = Testis weight × 100/Body weight.
[3] Hepatosomatic index = Liver weight × 100/Body weight.
[4] Thyroid epithelial height in microns.
[5] This group was autopsied after 7 weeks and the growth increments are not given since they are not comparable with the 8 week increments tabulated for the other groups. At the time of autopsy they had grown slightly more than the mock operated controls, but the differences were not statistically significant.
[6] In these groups the thyroids were hyperplastic owing to lack of an iodine supplement to the liver pablum diet. An independent investigation showed that the average thyroid epithelial height for aquarium males receiving iodinated liver pablum was 5.0 (7 fish, $s = 0.6$) (Pickford, 1953a).
[7] Length and weight changes are given for the eighth week but the autopsies were made after 9 weeks.

The fish were weighed and measured at weekly intervals. Standard rather than total length was taken, the fish being placed against an illuminated background to facilitate observation. The end of the vertebral column is marked by a bifurcation of the caudal vein which is readily visible under illumination. This procedure obviates errors in length measurement due to fraying of the tail margin. Scales were sampled at the beginning and end of the experiment; they were stained with alizarin red S and mounted in glycerine by the double coverslip method. At autopsy the livers and testes were weighed, the thyroid region and right head kidney (containing interrenal tissue) fixed in Bouin's fluid, and the brain exposed and fixed in Bouin. Completeness of hypophysectomy was deter-

mone preparations. "Growth data: 100 gm. hypophysectomized rats: (a) Organon 66450: 50 μg./day: av. net gain 22 gm. (b) 568 GH: 50 μg./day: av. net gain 16 gm.; 20 μg./day: av. net gain 13 gm. (c) 508 B: 20 μg./day: av. net gain 18 gm." He states that "These are essentially the responses to be expected of materials of unit activity or slightly better (1 gm. per day for 10 μg. per day)" Formal assays for other activities than growth have not been made. Dr. Wilhelmi states that "None of our preparations in the past has ever had any significant ACTH activity," and adds that "Your fish are a better indicator of contaminants than the standard assays are." Preparation 508 B was used in a previous experiment on killifish (Pickford, 1953b) and was found to be free of testis-stimulating and ACTH activity, but contained a trace of thyrotrophin.

mined by dissection under a binocular microscope but in one case there was evidence of residual thyrotrophic activity although no visible trace of the pituitary remained. The presence of a small persistent fragment, perhaps lodged in the scar tissue in the floor of the brain case, could only be determined by serial sections of the entire head and this was not attempted. One fish that was completely hypophysectomized had a tumorous swelling of the brain stem behind the optic nerves, perhaps resulting from an injury at the time of operation. Hemaglobin was estimated with the aid of the Tallqvist-Adams Hemoglobin Scale, used by medical practioners.

CONTROL EXPERIMENTS

Other experiments were in progress at the time of this investigation and, owing to a shortage of physiologically equilibrated hypophysectomized fish, it was decided to run the experiment without formal controls. It was felt that previous investigations (Pickford, 1953a, b) provided a background against which the results of the present investigation could be evaluated. Representative information taken from a previous investigation of the response to purified beef growth hormone (Pickford, 1953b) has been summarized in Table 1. Hypophysectomized killifish do not grow in length and the failure to grow is reflected in the absence of new circuli at the margin of the scales. Weight changes are erratic, depending on a variety of collateral causes, but saline injected hypophysectomized controls tend to show a progressive weight loss of greater or lesser severity. In respect to the internal organs, the testes of hypophysectomized fish are in complete regression. The livers are large, resembling those of autumnal wild fish, and are laden with fat and glycogen. In normal male fish the livers undergo a reduction in size and depletion of reserves in the spring and some preliminary investigations suggest that this response may be due to the release of androgens (Pickford, 1952a). The thyroids of hypophysectomized fish are quiescent, with a low epithelium and predominantly resting type colloid. A study of the adrenal cortical tissue of hypophysectomized fish was disappointing; no differences could be detected in comparison with unoperated and mock operated controls. The cells showed no signs of atrophy and granulation was normal. Hypophysectomized killifish tend to develop renal lithiasis, due to carbonate apatite concretions. A preliminary study of blood smears indicated that hypophysectomized fish were anemic.

RESULTS

Growth. Changes in weight and length during the course of the experiments are shown graphically in Figure 1. It will be seen that from the start of the experiment both groups began to increase in weight and length. A temporary weight decline at the end of the second week is probably due to the inclusion of chloromycetin in the food (0.5 gm./100 gm. liver-pablum) for a period of 5 days. This was necessary because a number of fish showed early symptoms of an unidentified infection which we have termed "pink disease" and which may be controlled either with chloromycetin or aureomycin.[5] There was no period of severe weight loss during the first weeks of

[5] Chloromycetin was donated by Parke, Davis and Co., through the courtesy of Dr. H. O. von Rosenberg. Calcium Aureomycin, which has proved extremely useful, was donated by the Lederle Laboratories, through the courtesy of Dr. D. W. Johnson and Dr. L. B. Tennison.

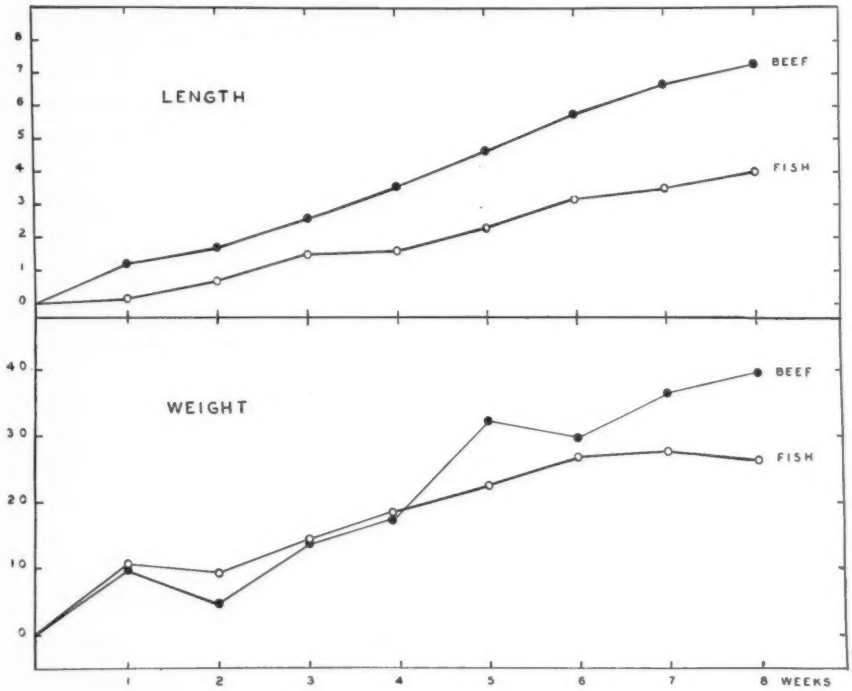

FIG. 1. Average percentage weight and length increments, at weekly intervals, of fish receiving fish growth hormone and fish receiving beef growth hormone.

the investigation such as was observed in the two earlier experiments with beef growth hormone.

Almost from the beginning of the experiment it became evident that fish receiving beef growth hormone were growing more rapidly than those receiving a similar dose of fish growth hormone. At the end of the experiment (Table 2) fish receiving fish growth hormone had increased on the average 26.3% in weight and 4.0% in length while fish receiving beef growth hormone had increased 39.5% in weight and 7.3% in length. Individual weight increments were very variable and even at the end of the experiment the difference between the means for the two groups is not statistically significant. The graph (Fig. 1) suggests that the weight increments were alike during the first few weeks but that after the end of the fourth week the fish receiving beef growth hormone showed a consistently greater gain. On the other hand the difference between the mean length increments became statistically significant at the end of the second week ($P = 0.1$–1%), when fish receiving beef growth hormone showed an average

TABLE 2. SUMMARY OF CONDITION OF FISH AT END OF EXPERIMENT: WEIGHT, LENGTH, SCALES, KIDNEYS, BLOOD

Fish	Growth:		Scales:		Kidney Stones	Hemoglobin[1] %	Pituitary
	Weight % incr.	Length % incr.	No. new circuli	New calcification			
Beef GH							
5.1	24.8	6.1	6.0	Abn.	Sl.	90	Abs.
5.2	34.4	7.7	5.5	Norm.	Sl.	60	Sm. nodule
5.3	27.9	5.5	3.5	None	Severe	70	Abs.
5.4	24.8	3.8	4.0	Abn.	Sl.	80	Abs
5.5	48.2	10.1	12.5	Norm.	Sl.	70	Abs.
5.6	64.1	10.8	10.0	Abn.	Sl.	70	Abs.
3.6	48.2	6.8	3.0	Abn.	Sl.	60	Abs.
Average	39.5	7.3	6.4	—	—	70	—
s[2]	16.1	2.5	3.6	—	—	10	—
Fish GH							
9.1	33.3	5.3	5.5	Abn.	Severe	70	Abs.
9.2	15.5	2.3	4.0	Abn.	Sl.	<30	Abs.
9.3	26.3	4.5	3.5	Norm.	Sl.	60	Abs.
9.4	30.7	4.4	2.0	None	Severe	70	Abs.
9.5	22.2	4.0	2.0	None	Severe	50	Abs.[3]
7.6	19.8	2.3	1.0	None	Severe	70	Abs.
1.6	37.5	5.0	3.0	None	Severe	60	Abs.[4]
Average	26.3	4.0	3.0	—	—	60	—
s	7.7	1.2	1.5	—	—	15	—
Significance of the difference							
P	5–10%	0.1–1%	2–5%	—	—	10–20%	—

[1] Tallqvist-Adams scale.
[2] Standard deviation for small numbers.
[3] Tumor behind optic nerves.
[4] Slight stimulation of thyroid indicates a persistent pituitary fragment not detected by dissection.

length increase of 1.7% while those receiving fish growth hormone had increased only 0.7%.

Scales. The addition of new circuli to the scales, determined by the method used previously (Pickford, 1953a and b), confirms the results on growth in length. Fish receiving fish growth hormone added on the average 3.0 new circuli whereas those receiving beef growth hormone added 6.4 (Table 2). The difference is statistically significant at the 2–5% level. Striking differences were observed in the character of the newly formed growth. In some fish (Table 2) this was completely normal and the resumption of new growth was marked by a perfectly typical annulus. In others the calcification was abnormal, as observed in the earlier experiments with beef growth hormone. In such cases the newly deposited calcification stains instensely with alizarin red S so that the scale appears under low magnification to have a brilliant red marginal zone. This is interpreted to mean that calcification was sparse, perhaps in the form of isolated crystals or small aggregations instead of the usual compact layer, since alizarin reacts with exposed surfaces and does not penetrate into the interior of a solid mass. Finally, a number of fish showed no calcification at all in the newly formed

marginal zone. New circuli are visible as ridges on the unstained fibrous layer which forms the newly outgrown margin of the scale. The significance of these defects of calcification must be considered in the light of other observations on the condition of the fish.

Reproductive System. One of the fish receiving fish growth hormone showed a very faint trace of nuptial coloration but this is of doubtful significance. The testes of all fish in this group were in total regression (average gonosomatic index 0.29, Table 3) as is normally the case in hypophysectomized males (Table 1). Fish receiving beef growth hormone developed more or less pronounced nuptial coloration at the end of the second week. This may be attributed to a trace of gonadotrophin, or possibly to intermedin, in the preparation (No. 568). An exactly similar experience was observed in an earlier experiment (Pickford, 1953b) with another growth hormone preparation. The colors faded away completely or persisted at a low level (Table 3) when the use of No. 568 was discontinued. However, at the end of the experiment the gonosomatic indices of these fish were

TABLE 3. SUMMARY OF CONDITION OF FISH AT END OF EXPERIMENT: NUPTIAL COLORS, TESTES, LIVER, THYROID

Fish	Nuptial colors[1]	GSI[2]	HSI[3]	Thyroid			Pituitary
				Av. cell ht., μ	% cells over 11 μ	Colloid[4]	
Beef GH							
5.1	−	0.63	4.5	6.8	8	R	Abs.
5.2	+	2.31	4.6	3.7	0	R	Sm. nodule
5.3	−	0.31	2.9	5.0	1	R	Abs.
5.4	−	0.31	3.7	6.7	4	R	Abs.
5.5	+	0.53	4.0	4.7	0	R	Abs.
5.6	±	0.68	4.5	5.3	1	R	Abs.
3.6	−	0.30	5.8	6.5	4	R	Abs.
Average	—	0.46[5]	4.3	5.5	2.5	—	—
s	—	0.18	0.9	1.1	—	—	—
Fish GH							
9.1	−	0.36	5.1	3.9	0	R	Abs.
9.2	−	0.30	5.0	3.8	0	R	Abs.
9.3	−	0.37	6.7	3.6	0	R	Abs.
9.4	−	0.25	6.7	4.0	0	R	Abs.
9.5	Trace	0.32	6.3	3.9	0	R	Abs.[6]
7.6	−	0.18	4.8	3.4	0	R	Abs.
1.6	−	0.23	4.2	4.8	2	SA	Abs.[7]
Average	—	0.29	5.6	3.9	0	—	—
s	—	0.07	1.0	0.4	—	—	—
Significance of the difference							
P	—	2–5%	2–5%	0.1–1%	—	—	—

[1] All negative at start.
[2] Gonosomatic index = Testes weight × 100/Body weight.
[3] Hepatosomatic index = Liver weight × 100/Body weight.
[4] R = resting: predominantly Azan red.
SA = slightly active: granular, Azan pink and blue.
[5] Excluding fish 5.2.
[6] Tumor behind optic nerves.
[7] Slight stimulation of thyroid indicates a persistent pituitary fragment not detected by dissection.

slightly higher than those of fish receiving fish growth hormone, the average being 0.49. The difference between the means for the two groups is statistically significant at the 2–5% level. On the basis of this experiment it is not possible to determine whether the beef growth hormone (Organon No. 66450) used during the last $4\frac{1}{2}$ weeks of the experiment contained a trace of gonadotrophin or whether the slight stimulation of the testes represents a persistent effect of the initial treatment with No. 568. None of the preparations elicited a spawning reflex (Pickford, 1952b).

One of the fish receiving beef growth hormone showed a definite enlargement of the testes (Table 3, fish 5.2, gonosomatic index 2.31). Autopsy revealed the presence of a minute persistant pituitary nodule. This specimen was excluded from the calculation of the average gonosomatic index for the beef growth hormone group. It has not been excluded from the other calculations because its growth was of the same order and the condition of the thyroid was similar to that of other members of its group. It is probable that the pituitary nodule contained only gonadotrophic cells.

Liver. The livers of fish receiving beef growth hormone were significantly smaller, at the 2–5% level, than those of fish receiving fish growth hormone (Table 3). The latter resemble those of hypophysectomized controls (Table 1). In 5 of the beef hormone group the liver color was pinkish or reddish brown whereas on the other two (fish 5.6 and 3.6) it was clay. The livers of fish receiving fish growth hormone varied from cream or buff to light brown. The change to a pinkish or reddish color is no doubt due to the depletion of fatty reserves, as observed in normal male fish in the spring (Pickford, 1953a).

Thyroid. With one exception, the fish receiving fish growth hormone had completely inactive thyroid glands (Table 3) resembling those of hypophysectomized controls (Table 1). Fish number 1.6 showed clear evidence of thyroid stimulation, at a low level. Although no persistant pituitary fragment could be found by dissection it is suspected that some thyrotrophic cells remained, perhaps within the scar tissue in the roof of the mouth. It is probably significant that the growth of this fish was good, although not greater than that of fish number 9.1 in which the thyroid was inactive.

In fish receiving beef growth hormone, on the other hand, the thyroids were maintained at a normal level similar to that observed in unoperated fish under aquarium conditions (Pickford, 1953a). Similar results were obtained previously with preparation No. 508B (Wilhelmi). It appears that Fundulus is extremely sensitive to minute traces of thyrotrophin. The average cell height was 5.5μ and in 5 out of the 7 fish some follicles contained cells that were strongly stimulated, 12μ or more in height (Table 3). On the other hand the colloid was predominantly resting, Azan red and homogeneous in character. The difference in mean cell height between the

fish and beef growth hormone groups is highly statistically significant.

Interrenal. The interrenal or Giacomini tissue of hypophysectomized Fundulus is not demonstrably different from that of unoperated controls (Pickford, 1953a). According to unpublished observations injections of adrenocorticotrophin into hypophysectomized fish of this species induces some proliferation of the interrenal tissue. In the present experiments no differences were observed between fish receiving fish growth hormone and those receiving beef growth hormone. The interrenal tissue was unstimulated and appeared completely normal. This cannot be attributed to maintenance by traces of ACTH since the adrenal tissue of hypophysectomized controls shows no signs of atrophy. It was observed that Bouin's fluid, which was used in this experiment to promote easier sectioning, gave very poor fixation of the adrenal tissue. The cells were often separated from each other by spaces and frequently the whole Giacomini layer, lining the wall of the posterior cardinal vein, was completely disrupted. Such effects have never been observed after fixation in Helly's fluid unless a fish had died before autospy so that autolysis had set in.

Kidneys. The incidence of kidney stones in hypophysectomized Fundulus has been described previously (Pickford, 1953a, b) and it was shown by Dr. Clifford B. Frondel (1953) that the material is composed largely of hydrated carbonate apatite. In the present experiment conspicuous urinary calculi were observed macroscopically at autopsy in a number of fish. Five of the fish hormone group were affected in this manner but only one case was observed among the beef growth hormone group (Table 2). However, kidney sections showed evidence of occasional small concretions even in those fish that were macroscopically normal. There is a high order of correlation between the incidence of severe renal lithiasis and the failure of new calcification on the scales.

Blood. Nearly all the fish were somewhat anemic (Table 2). This is concordant with previous findings for hypophysectomized fish. Normal values for wild males average 80% and a group of saline injected hypophysectomized males averaged 68% (unpublished observations). The slight difference between the beef and fish growth hormone groups is not statistically significant but could, if it were, be attributed to the stimulation of the thyriods. Kalashnikov and Skadovsky (1940, 1948) claim that treatment with thyroxin (by injection) or thyroidin (per os) prevents the development of anemia and degeneration of the ova that occurs when female sevriuga, Acipenser stellatus, captured on their upstream spawning migration are taken into captivity for piscicultural purposes. The investigations were primarily concerned with artificial induction of spawning, and prevention of the degeneration of the gonads; the statements about the prevention of anemia are not accompanied by supporting experimental data. In these,

and in two earlier investigations (Kalashnikov, 1939a, b), it was claimed that injections of fresh pituitary extract resulted in a less pronounced anemia.[6]

DISCUSSION

The results presented above permit the following conclusions. The purified fish growth hormone preparation F80GH was completely free of gonadotrophins. The testes remained unstimulated and no spawning reflex was elicited. The latter response can be obtained even in hypophysectomized killifish with certain fish pituitary preparations, and has been attributed to the luteinizing hormone (Pickford, 1952b). Preparation F80GH was also free from thyrotrophin and adrenocorticotrophin. The only collateral activity associated with this fraction was the melanophore concentrating agent which is present in all fish pituitary fractions that we have so far investigated and which induces a transitory pallor after each injection.

The beef growth hormone fractions, like other mammalian anterior lobe preparations that have so far been tested by us, lack the melanophore concentrating agent. On the other hand there is evidence of thyrotrophic activity and, in No. 568 (Wilhelmi), and possibly also in Organon No. 66450, there is a trace of follicle stimulating activity, witnessed by the induction of nuptial coloration and slight enlargement of the testes. There was no evidence of corticotrophic activity.

In comparing the growth responses of the two groups it is obvious that the fish growth hormone preparation was less effective. Weight changes are very variable and their interpretation is complicated by collateral causes. The best criterion of growth is the increase in length and, on this basis, the fish growth hormone had only 55% of the activity of the beef growth hormone. Since the latter contained thyrotrophic activity it may be asked whether the difference was due to this cause or to a lower growth promoting activity of the fish growth hormone *per se*. According to Wilhelmi (1954) the fish growth hormone contained a second, apparently inactive component, perhaps of the order of 25%. This would largely account for the lower order of response. On the other hand the collateral role of the thyroid cannot be excluded and it is hoped to make further experiments in which the growth of fish receiving fish growth hormone is compared with that of fish receiving, in addition, either thyrotrophin or a thyroxin supplement.

In comparison with previous experiments (Pickford and Thompson, 1948; Pickford, 1953b) two further points may be noticed. Neither with beef growth hormone nor with fish growth hormone was there any appreci-

[6] I am greatly indebted to Dr. A. Petrunkevitch, of the Osborn Zoological Laboratory, Yale University, who has spent many hours in giving me oral translations of the Russian text of these and other articles.

able delay in the initiation of the response, in particular there was no period of weight loss preceding the renewal of growth. This observation eliminates the hypothesis that the initial period of weight loss is due to an unfavorable reaction to the mammalian preparation. It is believed that the elimination of the period of weight loss can be attributed to improved experimental conditions, in particular to the addition of a dietary supplement of frozen daphnia. A control group receiving saline injections, run in another series of experiments but under identical conditions, showed only a comparatively small wieght loss, −6.1% after one month. This is very different from the severe weight loss observed in controls in the earlier experiment, −13.5% after one month, −17.5% after two months (Pickford, 1953b). The hypothesis regarding improved experimental conditions may also explain the much greater growth response that was elicited in this experiment as compared with the earlier one (Pickford, 1953b). In the latter experiment hypophysectomized fish receiving beef growth hormone increased 14.8% in weight and 4.2% in length after 8 weeks, whereas in the present experiment they increased 39.5% in weight and 7.3% in length during the same period of time. It is true that different beef growth hormone preparations were used in the two experiments, but they had the same order of activity when tested on rats and both contained a similar trace of thyrotrophin.[7]

The fact that fish show no antagonism towards beef growth hormone is in striking contrast to reciprocal experiments reported by Jampolsky and Hoar (1953) with crude preparations of fish growth hormone and more recently by Wilhelmi (1954) with the highly purified fractions used in the present experiments. These authors agree that rats show at best only a transitory weight increase when injected with fish growth hormone preparations, followed by a progressive weight loss.

The present experiments throw no light on the comparatively high dosage level that is required to induce a growth response in fish. Fish growth hormone is not more active than beef growth hormone and with both types of preparation a dosage level of 10 μg./ gm. weight, thrice weekly, is necessary to elicit a satisfactory response. A series of investigations on the effects of temperature, still in progress, appears to indicate that higher temperatures do not establish a greater response and that 20°C. may be optimal for this species. If this is true the lower sensitivity of fish must be attributed to inherent limitations in the growth capacity of the organism.

Another interesting problem concerns the failure of calcification of new growth on the scales. The results are not uniform and, perhaps by chance, a larger number of fish in the fish growth hormone group show defective

[7] As tested on fish, conventional assays are not available (see footnote 4).

calcification. However, one fish in the beef growth hormone group was severely affected and therefore the condition cannot be related to the difference in the activity of the thyroids. On the other hand there is a high correlation between the incidence of severe renal lithiasis and the failure of calcification. It is also clear that the disturbance of calcium metabolism in the hypophysectomized fish, witnessed by these defects, is not ameliorated by treatment with growth hormone. Further studies are under consideration.

The smaller size of the livers in the beef growth hormone group is possibly related to the stimulation of the testes. Preliminary experiments with methyl testosterone and thyroxin (Pickford, 1952a) indicated that the male hormone might be responsible for the reduction of liver size in male fish during the breeding season but the matter is still under investigation.

SUMMARY

Hypophysectomized male killifish (Fundulus heteroclitus) at 20°C. were injected thrice weekly for 8 weeks with purified fish growth hormone (F80GH, Wilhelmi) and purified beef growth hormone (568, Wilhelmi, 6 times; 508B, Wilhelmi, 5 times; Organon 66450, 14 times) at a dosage level of 10 μg. per gram weight. The fish growth hormone was free from thyrotrophin, gonadotrophins, and adrenocorticotrophin, but contained the melanophore concentrating agent. Beef growth hormone No. 568 stimulated the nuptial coloration and was discontinued; No. 508B did not. Organon 66450 maintained the thyroids at a normal level and may have contained a trace of gonadotrophin; there was no stimulation of the adrenal cortical tissue and the mammalian preparations were free from melanophore concentrating activity. Average weight and length increments at the end of the experiment were: fish growth hormone, 26.3% and 4.0%; beef growth hormone, 39.5% and 7.3%. There was no initial period of weight loss. New growth was elicited at the margin of the scales but calcification was frequently defective or lacking. There is a high correlation between the incidence of severe carbonate apatite renal lithiasis and lack of new calcification. Livers of fish receiving beef growth hormone were significantly smaller than those of fish receiving fish growth hormone, possibly associated with the slight stimulation of the testes. All fish were slightly anemic. The lesser response to fish growth hormone may be due to the presence of an inactive component or correlated with the total lack of thyroid stimulation.

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THE INFLUENCE OF 3-METHYLGLUCOSE ON THE HYPERTENSION AND CARDIOVASCULAR-RENAL CHANGES ELICITED BY DESOXYCORTICOSTERONE ACETATE IN THE RAT

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IT HAS now been well established in the rat, cat and dog that 3-methylglucose, a mono-methyl ether of glucose, is rapidly absorbed from the gastro-intestinal tract, metabolized slowly and rapidly excreted in the urine (Czaky, 1942; Campbell, 1949; Campbell and Davson, 1949; Young, 1950; Skelton, McConkey, Souch and Grant, 1951). When a sufficient quantity of this compound is present in the urine, for example following its administration as 20 per cent of the diet, an osmotic diuresis with marked loss of sodium and a concomitant polydipsia are produced (Skelton, McConkey, Souch and Grant, 1951).

The importance of the sodium ion in the development of hypertension and cardiovascular renal lesions during desoxycorticosterone acetate (DCA) treatment has now been demonstrated in several species (Selye and Stone, 1943; Selye, Mintzberg and Rowley, 1945; Selye, Stone, Timiras and Schaffenburg, 1949). In this connection it has also been shown that the administration of either an acidifying salt such as ammonium chloride (Selye, Hall and Rowley, 1945), or the forced feeding of a 25 per cent dextrose solution (Berman, Hay and Selye, 1945) can substantially inhibit the functional and morphologic effects of prolonged DCA administration in the rat. This has been presumed to be the result of the increased urinary loss of sodium induced by these substances. In view of the ability of 3-methylglucose to produce both polydipsia and diuresis, the following experiments were performed to study the effects of this compound on DCA overdosage in the rat from two standpoints: First, as a means of potentiating the actions of the steroid by increasing the intake of sodium chloride in the drinking fluid, and second, as an inhibitor of its toxic effects by increasing the urinary loss of sodium.

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MATERIALS AND METHODS

Experiment 1. Thirty-two female hooded rats weighing approximately 120 gm. were divided into four groups of 8 rats each, unilaterally nephrectomized and given 1% sodium chloride to drink *ad libitum*. Group I served as controls. The rats of Group II received daily subcutaneous injections of 0.5 mg. of DCA as a microcrystalline suspension in 0.1 ml. of distilled water. The animals of Group III received 3-methylglucose as 20 per cent of their diet (Skelton, McConkey, Souch and Grant, 1951) and the rats of Group IV were treated with both DCA and 3-methylglucose as described for Groups II and III respectively. Throughout the experiment the saline consumption for each rat was measured daily and blood pressure was determined weekly by a modification of the tail plethysmographic method of Williams, Harrison and Grollman (1939). At the termination of the experiment all rats were weighed and killed by exsanguination. The kidney, heart, liver, adrenals and thymus were removed and fixed in Bouins solution for 24–48 hours before being weighed on a chainomatic balance. These organs were subsequently sectioned at 6 microns and stained with hematoxylin and eosin for microscopic study.

Experiment 2. Thirty female hooded rats weighing approximately 120 gm. were unilaterally nephrectomized, given 1% sodium chloride to drink, divided into 3 groups of 10 rats each and treated as follows: The rats of Group I received no further treatment. The rats of Group II were given daily subcutaneous injections of 2.5 mg. of DCA as a microcrystalline suspension in distilled water and the animals of Group III were treated with DCA in a manner identical to Group II, and in addition received 3-methylglucose as 20% of their diet.

All rats were maintained in metabolism cages and throughout the experiment their saline consumption was measured daily. The saline intake of the animals of Group III was carefully adjusted so that it paralleled as closely as possible the amount consumed voluntarily by the animals of Group II. Inasmuch as the drinking fluid was the sole source of sodium, the intake of this ion by the rats of both groups was closely approximated. The individual urine volumes for each group of rats were also determined daily, and the blood pressures were measured weekly as in Experiment 1. The experiment was terminated after 6 weeks and the same procedures carried out as in Experiment 1.

EXPERIMENTAL OBSERVATIONS

Experiment 1. The average saline consumption and blood pressures for each group are graphically presented in Figure 1. It is seen in the upper part of the figure that the sodium chloride consumption of the DCA-treated rats remained almost identical to that of the controls, while a very significant increase occurred in both groups receiving 3-methylglucose in their diet. The intake of the rats treated with both DCA and 3-methylglucose, however, was appreciably greater (93 ml. daily average for each rat) than the intake of the rats receiving 3-methylglucose only (68 ml. daily average for each rat). The changes in the blood pressure of the various groups were remarkably parallel to the changes observed in the saline intake. Thus a statistically significant degree ($P<0.01$) of hypertension was observed only in the rats of Group IV, while the slight elevation of blood pressure which was present terminally in the animals treated with either

TABLE 1. THE INFLUENCE OF THE ADMINISTRATION OF 3-METHYLGLUCOSE AS 20 PER CENT OF THE DIET ON THE BODY AND ORGAN WEIGHTS OF RATS TREATED WITH DCA AND ALLOWED 1 PER CENT NaCl TO DRINK AD LIBITUM

Group and treatment	Number of rats	Body weight in gm.		Organ weights in mgm. per 100 gm. of initial body weight				
		Initial	Final	Kidney	Heart	Liver	Thymus	Adrenals
I Control	8	131±4	146±5	519±18	443±10	5230±187	100±9	24.9±2.8
II DCA	8	135±4	150±3	720±25	480±16	5500±253	119±10	22.0±1.8
III 3-Methylglucose	5	139±4	165±3	620±47	441±11	3970±183	126±10	26.8±1.9
IV DCA + 3-Methylglucose	6	145±2	160±3	776±73	495±23	3680±188	92±2.5	25.0±2.6

All values accompanied by their standard error.

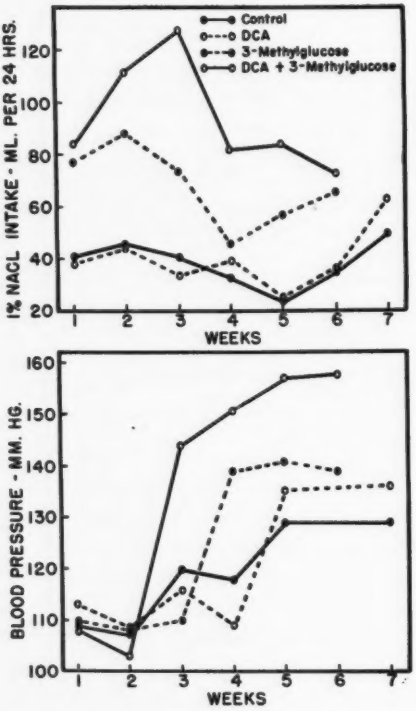

FIG. 1. The influence of the administration of 3-methylglucose as 20% of the diet on the blood pressure and 1% NaCl intake of rats treated with DCA.

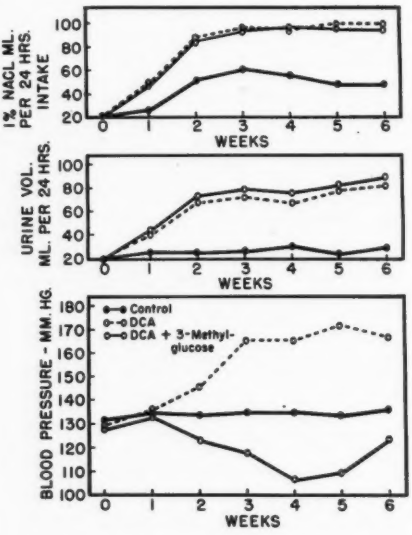

FIG. 2. The influence of the administration of 3-methylglucose as 20% of the diet on the blood pressure and urine excretion of rats treated with DCA when the 1% NaCl intake of experimental groups is equalized.

DCA or 3-methylglucose (Groups II and III respectively) was not statistically significant.

The body and organ weight changes are presented in Table 1. The organ weights are expressed as mgm. per 100 gm. of initial body weight in order to compensate for the slightly irregular weight gains of the various groups. During the experiment a total of 5 rats died from Groups II and IV. It is of interest that significant ($P < 0.01$) renal hypertrophy occurred in both Group II and IV without apparent relationship to hypertension, since a significant elevation of blood pressure occurred in the later group only. On the other hand the cardiac weights appeared to be closely related to the degree of hypertension inasmuch as significant ($P < 0.05$) hypertrophy was present in the rats of Group IV only. An unexpected observation was the markedly significant ($P < 0.01$) atrophy of the liver in the rats of Groups II and IV all of which were fed 3-methol glucose in their diet.

Microscopic examination of the kidneys of rats treated with either DCA (Fig. 3) or 3-methylglucose (Fig. 4) showed minimal thickening of the wall of the glomerular tufts and no vascular or tubular abnormalities. On the other hand the kidneys of rats treated with both DCA and 3-methylglucose (Fig. 5) showed marked enlargement and hyalinization of glomeruli with peri-glomerular fibroblastic proliferation, hyaline arterial and arteriolonephrosclerosis, dilatation of renal tubules and extensive formation of hyaline and granular casts. No lesions were observed in the hearts of either the control rats or those treated with 3-methylglucose (Fig. 7), but small areas of degeneration of myocardial fibers with fibroblastic proliferation and infiltration of Anitschkow myocytes and mononuclear cells were present in the hearts of the animals treated with DCA (Fig. 6). In contrast the cardiac lesions in the rats receiving both DCA and 3-methylglucose (Fig. 8) were more severe, consisting of numerous hyalinized small arterioles as well as changes similar to those described in the hearts of the rats of Group II. The greater severity of the pathologic

TABLE 2. THE INFLUENCE OF THE ADMINISTRATION OF 3-METHYLGLUCOSE AS 20 PER CENT OF THE DIET ON THE BODY AND ORGAN WEIGHTS OF RATS TREATED WITH DCA WHEN THE 1 PER CENT NaCl INTAKE OF EXPERIMENTAL GROUPS IS EQUALIZED

Group and treatment	Number of rats	Body weight in gm.		Organ weights in mgm. per 100 gm. of initial body weight				
		Initial	Final	Kidney	Heart	Liver	Thymus	Adrenals
I Control	10	116±2	185±2	785±35	540±12	5620±182	192±12	37.5±1.1
II DCA	6	120±3	173±9	1550±240	678±43	6260±520	189±15	28.8±2.4
III DCA + 3-Methylglucose	8	118±2	118±7	880± 70	408±18	4180±270	52± 7	28.8±1.7

All values accompanied by their standard error.

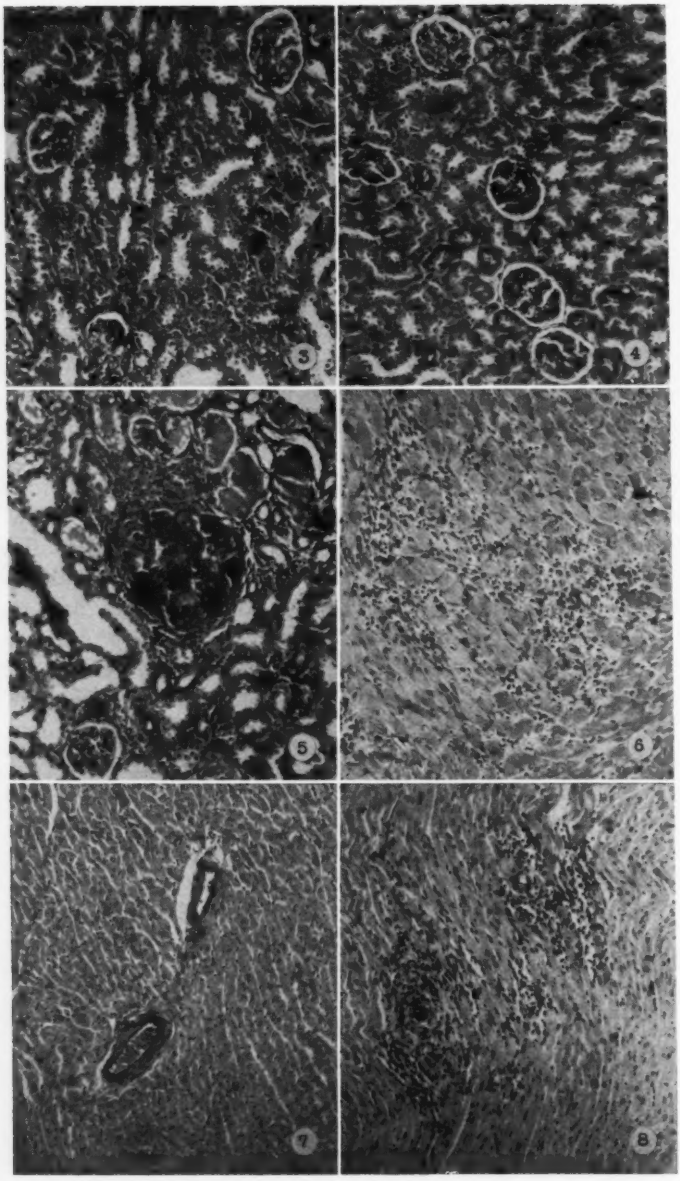

PLATE I

changes in Group IV was also indicated by the presence of hyalinized pancreatic arterioles surrounded by marked perivascular fibroblastic proliferation and fibrinoid material.

Experiment 2. A graphic presentation of the saline intake, urine output and blood pressures is contained in Figure 2. The increased consumption of the 1% sodium chloride solution by the rats of Group II and the almost identical regulated intake of the animals in Group III is clearly evident. A similar increase in the urinary output of the rats of both experimental groups is also shown in Figure 1, but it is noteworthy that the volume excreted by the rats treated with DCA and 3-methylglucose (Group III) was consistently greater than that excreted by the rats receiving DCA alone. The lower part of the figure shows that the blood pressure of the DCA-treated animals reached hypertensive levels after 3 weeks, in contrast to the persistent hypotension of the rats treated with DCA plus 3-methylglucose.

Table 2 shows that 6 rats from Groups II and III died during the experiment and that the growth of the remaining rats of Group III was considerably retarded, an observation which again necessitated expression of the organ weights in the remainder of the table as mg. per 100 gm. of initial body weight. It will be noted that significant renal and cardiac hypertrophy ($P < 0.01$) was present in the rats treated with DCA (Group II) in striking contrast to the almost complete absence of renal hypertrophy and significant cardiac atrophy ($P < 0.01$) in the rats of Group III. Despite the absence of hypertrophy of the adrenals ($P < 0.05$), the atrophy of the thymus ($P < 0.01$) in Group III suggests some adrenocortical activation in these rats. Atrophy of the liver similar to that noted in experiment 1 was also observed in the rats treated with 3-methylglucose in this experiment (Group III).

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FIG. 3. Kidney of rat treated with DCA showing essentially normal appearance of glomeruli and tubules. H. & E. ×150.

FIG. 4. Kidney of rat treated with 3-methylglucose also showing normal appearance. H. & E. ×150.

FIG. 5. Kidney of rat treated with both DCA and 3-methylglucose showing marked hyalinization of one large glomerulus, tubular dilatation and cast formation. Note the small hyalinized arteriole adjacent to the uninvolved glomerulus. H. & E. ×150.

FIG. 6. A focal area of degeneration of myocardial fibers with fibroblastic prolifieration and mononuclear and Anitschkow cell infiltration in a rat treated with DCA alone. H. & E. ×150.

FIG. 7. Normal appearance of the myocardium and arterioles in a rat treated with 3-methylglucose. H. & E. ×150.

FIG. 8. Potentiation afforded by combined treatment of DCA and 3-methylglucose is illustrated by the more marked degeneration of myocardial fibers, arteriolosclerosis and the presence of slight perivascular fibrinoid material. H. & E. ×150.

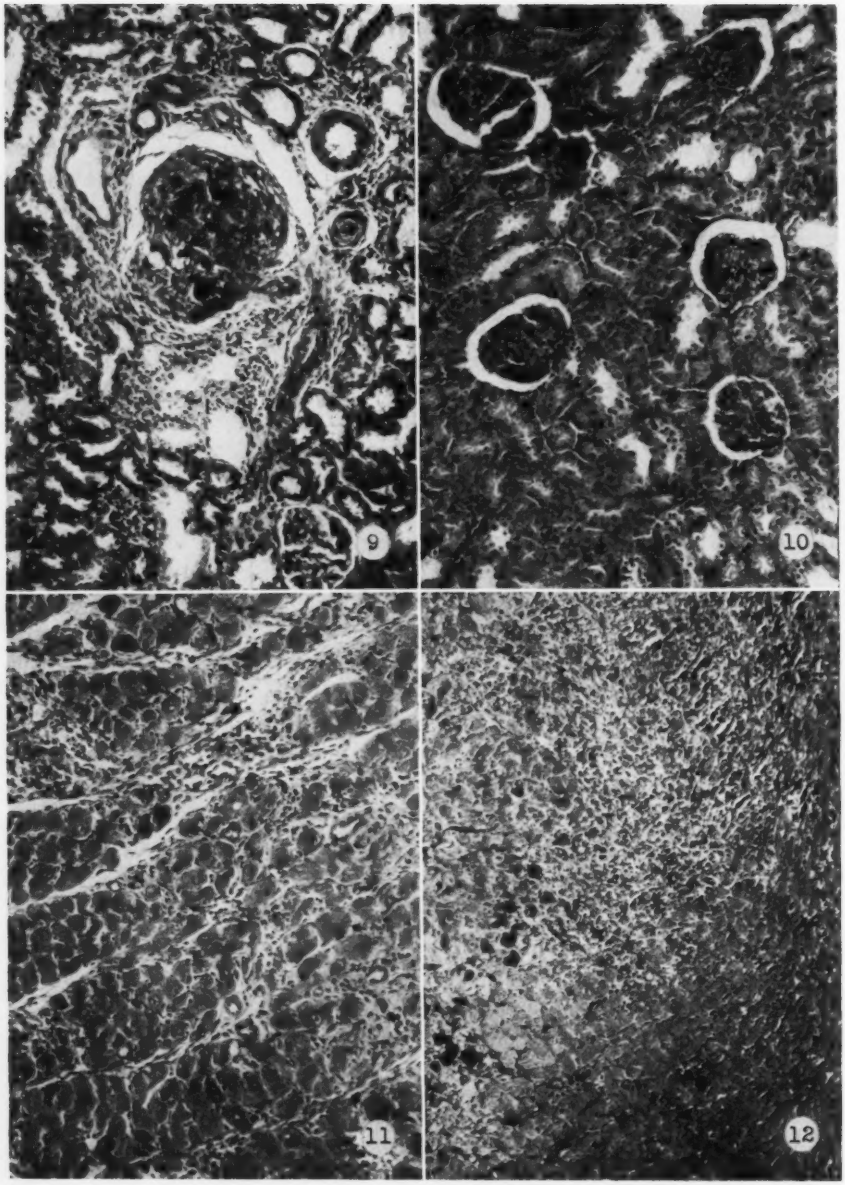

PLATE II

The kidneys of all rats in Group II showed marked glomerular hyalinization and scarring, arteriolonephrosclerosis, tubular dilatation and hyaline cast formation (Fig. 9). On the other hand there was almost total absence of such changes in the kidneys of the rats of Group III (Fig. 10). Such divergent microscopic changes were not observed in the hearts, since almost equally severe hyaline degeneration of myocardial fibers, fibroblastic proliferation, arteriolar hyalinization, and infiltration of mononuclear cells and Anitschkow myocytes were present in all of the sections studied from both Groups II and III (Figs. 11 and 12).

DISCUSSION

Experiments in which rats were fed 3-methylglucose as 20% of their diet and given water to drink for one year failed to demonstrate any toxic manifestations of this compound, despite the presence of prolonged polydipsia and diuresis (Skelton, McConkey, Souch and Grant, 1951). The production of marked polydipsia and diuresis in the rats of the first experiment which were fed 3-methylglucose and given saline solution *ad libitum* confirms the previous studies, but the concomitant finding of marked atrophy of the liver had not been previously observed to accompany 3-methylglucose administration. The cause of this hepatic atrophy and its significance are as yet unknown, but it should be noted that the present experiments differ from those of the previous report in that the rats drank 1% sodium chloride instead of water and were sacrificed during their active growth period. However, if either of these factors alone were of fundamental importance in the production of the hepatic atrophy one would expect a similar finding in the rats treated with DCA in the second experiment, and such was not the case. Consequently it is suggested that, since 3-methylglucose is not metabolized, the normal glycogen stores of the liver are mobilized with resultant reduction in size of the organ. It may fur-

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FIG. 9. Marked renal lesions in rat treated with DCA and allowed 1% NaCl *ad libitum*. Note the typical glomerular hyalinization, tubular dilatation and arteriolonephrosclerosis. H. & E. ×150.

FIG. 10. Kidney of rat treated with DCA and 3-methylglucose in which the 1% NaCl consumption was equalized with that of control rat treated with DCA alone. Note the complete absence of pathological changes in this organ. H. & E. ×150.

FIG. 11. Hyaline degeneration of myocardial fibers, fibrosis and infiltration of mononuclear cells in the heart of the same rat whose kidney is shown in Fig. 9. H. & E. ×150.

FIG. 12. Extensive hyaline degeneration of myocardial fibers with fibroblastic proliferation and infiltration of inflammatory cells in the heart of the same rat whose kidney is shown in Fig. 10. It is of interest that the myocardial lesions in this particular animal are more severe than in the control rat treated with DCA (Fig. 11), although other rats of the group showed lesions of essentially the same severity as in Fig. 11. H. & E. ×150.

ther be related simply to growth inhibition since Quimby (1948) has observed a reduction in liver size in rats as a result of starvation during their active growth period.

The development of statistically significant hypertension, cardiac hypertrophy, as well as renal and cardiac lesions in the rats of experiment 1 which were treated with both DCA and 3-methylglucose, and the absence of these findings in the animals treated with either substance alone, indicates the potentiation of known DCA effects which was afforded by the administration of this glucose derivative. The concomitant increase in the saline consumption of the rats receiving the combined treatment again confirms the importance of the sodium ion in the genesis of the above changes. On the other hand neither increased sodium consumption nor hypertension seems to be of fundamental importance in the renotrophic action of this steroid since almost identical renal hypertrophy was observed in both the normotensive, DCA-treated rats and the hypertensive rats receiving DCA plus 3-methylglucose. Additional evidence in support of this observation has been reported by Masson and Beland (1943).

When the sodium chloride consumption was equated in rats treated with DCA alone and DCA plus 3-methylglucose as in experiment 2, the hypertension, cardiac and renal hypertrophy were inhibited in those animals receiving the combined treatment. It was considered of some interest, however, that the microscopic observations in the heart and kidneys did not entirely parallel either the blood pressure levels or the gross findings. Thus, the nephrosclerotic renal lesions produced by DCA were almost entirely prevented by the administration of 3-methylglucose, but the cardiac lesions appeared equally as severe in the rats treated with both 3-methyglucose and DCA as in those animals receiving the steroid alone.

What possible explanations are there for these latter observations? It will be recalled that the average 24 hour urine excretion was slightly greater in the rats receiving combined treatment than it was in the rats receiving DCA alone, a fact which suggests a greater loss of sodium in the former animals since it has been shown that excretion of 3-methylglucose in the urine is accompanied by increased loss of sodium (Skelton, McConkey, Souch and Grant, 1951). Such an increased excretion of sodium in this group could explain the inhibition of hypertension, renal and cardiac hypertrophy and the absence of nephrosclerosis, but is somewhat inconsistent with the continued presence of cardiac lesions. Perhaps this unexpected observation is related to a relative potassium deficiency in these rats since both saline infusions (Stewart and Rourke, 1942) and 3-methylglucose administration (Skelton, McConkey, Souch and Grant, 1951) have been shown to induce some loss of potassium. Furthermore cardiac lesions in potassium deficiency have been repeatedly described (Schrader, Prickett and Salmon,

1937; Thomas, Mylon and Winternitz, 1940; Follis, Orent-Keiles and McCollum, 1942; Kornberg and Endicott, 1946, Smith, Black-Schaffer and Lasater, 1950; French, 1952) and have been likened by Peschel, Black-Schaffer and Schlager (1951) to the changes produced by DCA administration. Certainly there are similarities, but a characteristic lesion in the hearts of rats treated with DCA which is not present in potassium deficient animals, is hyalinization of arterioles with perivascular fibrinoid material and fibroblastic proliferation (Selye, 1950; Masson, Hazard, Corcoran and Page, 1950). It is the opinion of the author that the hyaline degeneration of myocardial fibers in the rats treated with both DCA and 3-methylglucose in the second experiment is quite suggestive of potassium deficiency, but the vascular changes and surrounding fibrinoid degeneration are more characteristic of the lesions induced by DCA. The problem is made even more complex by the recent experiments of Cannon, Frazier and Hughes (1953) who have presented evidence that the sodium ion acts as a noxious agent in potassium deficiency by virtue of its inactivation of intracellular enzymes thus leading to degeneration and death of the myocardial fiber.

On the other hand Rosenberg, Woodbury and Sayers (1952) have shown that both cortisone and ACTH can inhibit the changes of DCA over-dosage in the rat. The thymus atrophy in the rats receiving combined DCA and 3-methylglucose treatment in the second experiment suggests that the inhibiting mechanism of 3-methylglucose might be by way of an increased endogenous production of gluco-corticoids, but the present author has observed more marked activation of the adrenal cortex than that which is present in this experiment without any inhibition of the changes induced by DCA. In agreement with other investigators Rosenberg, Woodbury and Sayers (1952) observed that DCA did not regularly induce pathologic changes from one experiment to another, but when such changes were produced they were uniformly present in the DCA-treated animals of any given experiment. Similarly, uniform pathologic changes in the kidneys, heart and pancreas were observed in the rats of Group IV in the first experiment, but the absence of renal lesions and the persistence of cardiac changes in the rats treated with both DCA and 3-methylglucose (Group III) in the second experiment represents the first time that such pronounced dissociation of pathologic changes has been reported. At the present time no definite explanation can be given for these observations, but the most fruitful possibility seems to be that the administration of 3-methylglucose induced diuresis of both sodium and potassium, and that the loss of the former ion was sufficient to inhibit the development of hypertension and renal changes while the loss of the latter ion at least contributed to the persistence of the cardiac lesions.

SUMMARY

A diet containing 20% 3-methylglucose was fed to unilaterally nephrectomized rats which were also treated with DCA and sodium chloride. When the sodium chloride was given *ad libitum* 3-methylglucose produced potentiation of the cardiovascular-renal effects of DCA as shown by the blood pressure changes, cardiac hypertrophy and histologic changes in the kidney, heart and pancreatic arterioles. This effect of 3-methylglucose is felt to be the result of the increased consumption of sodium chloride induced by this compound. On the other hand when the sodium chloride consumption of animals treated with DCA alone and with DCA plus 3-methylglucose was equalized, there was almost total inhibition of the DCA effects by the 3-methylglucose. This was evidenced by hypotension, cardiac atrophy and the absence of renal hypertrophy and histologic changes, although there was no concomitant inhibition of the cardiac lesions. Under the present experimental conditions, 3-methylglucose also induced marked atrophy of the liver. Possible explanations for this effect of the compound are discussed, but no conclusions can presently be drawn.

These experiments further reiterate the fundamental importance of the sodium ion in the genesis of the changes produced by DCA overdosage in the rat, and also show that the various effects of this steroid can be dissociated. Additional experiments are projected to study conditions under which dissociation of DCA changes are produced with particular reference to the role of potassium.

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BODY TEMPERATURE, THYROID AND ADRENAL CORTEX OF HAMSTERS DURING COLD EXPOSURE AND HIBERNATION, WITH COMPARISONS TO RATS[1]

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SINCE the early part of the twentieth century, many studies have been made to assess the activity of the endocrine glands in mammals before and after entrance into hibernation (Kayser, 1950, 1953). It has been frequently reported for the hibernating species that there is an involution of the anterior pituitary, thyroid and adrenal cortex during the autumn and that this condition persists during hibernation [e.g., marmot (*Marmota*), Coninx-Girardet, 1927; 13-lined ground squirrel (*Citellus*), Foster, Foster and Meyer, 1939; hedgehog (*Erinaceus*), Skowron and Zajaczek, 1947; European hamster (*Cricetus*) and dormice (*Myoxus*, *Muscardinus*), Kayser and Aron, 1950]. Kayser supports Adler's (1926) hypothesis that hibernation is a direct result of a polyglandular depression in the autumn which reduces the ability of the animal to maintain its normal homeothermic adjustment when exposed to cold.

The observations which form the basis for the Adler-Kayser hypothesis were made for the most part on animals which were killed in the field. The experiments reported here were conducted under controlled laboratory conditions. Furthermore, the observations were made in the spring and summer rather than during the autumn and winter. We have compared the adrenal cortex and the thyroid gland in Syrian hamsters maintained at ordinary temperatures, in hamsters exposed to the cold for various periods but not hibernating, and in hamsters that had been hibernating in the cold for different lengths of time. We also compared the responses of these glands in hamsters and in laboratory rats exposed to identical cold stresses. In addition, some experiments were made to test the responses of the thyroid and adrenal cortex of hamsters to stimuli other than cold. The thyroid and adrenal glands were studied by histological and histochemical procedures which have proved useful in assessing their physiological activity (Dempsey and Singer, 1946; Greep and Deane, 1949).

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MATERIAL AND METHODS

Young adult Syrian hamsters (*Mesocricetus auratus*), weighing 80 to 130 gm., and young adult albino rats of the Sprague-Dawley strain, weighing 145 to 210 gm., were used. All animals were supplied with Purina laboratory chow and water *ad libitum*. The experiments were conducted between the months of April and October.

The control animals were maintained in individual cages in the animal stock room (temperature, 23° C.±5). The room was illuminated artificially for approximately 8 hours daily during all of the year, and received some additional light during the longer summer days. The animals exposed to a temperature of 5° C.±2 were housed in similar individual cages and received 8 hours of illumination a day.

Experiment 1. Twenty female hamsters were given shavings for nesting material and observed daily in the cold room to determine if they had entered hibernation. Eight of the animals were killed at various times before they hibernated (May to Sept.), the rest hibernated and were killed between 1 and 83 days after their first day of hibernation (July to Oct.).[3] Eight control hamsters were killed between May and October. Body temperatures were taken just before death. A Micromax[4] thermoelectric recorder calibrated to 0.25° C. was used, and the thermocouple was placed in the most caudal part of the cheek pouch close to the body.

Experiment 2. In a preliminary experiment (April to June), hamsters and rats of both sexes were exposed to cold with shavings for bedding. The animals were killed after 2 to 18 days, and their adrenal and thyroid glands studied. Since the rats exposed to these conditions showed minimal stimulation of these glands, a second experiment was performed from July to October under more rigorous conditions. Twenty male hamsters and 21 male rats were exposed to cold in suspended wire-floored cages *without* bedding. Groups were killed after 3 to 21 days. There were 5 control hamsters and 5 control rats. Body temperatures were taken daily for both species, using deep rectal temperatures for the rats.

Experiment 3. To test the thyrotropic potency of the hamster pituitary, 7 hamsters were given 0.1% thiouracil in the drinking water and 0.2% in ground food. They were killed from 1 week to 2 months later. The thyroids were weighed, as were those of 4 control animals. One experimental and one control gland were analyzed for I_2 content by Dr. Edwin B. Astwood.

Experiment 4. To test the corticotropic response of the hamster pituitary, daily intramuscular injections were made of epinephrine[5] in peanut oil, 0.1 ml. of a 1:2500 solution, 0.4 mg./kilo (Chan, 1950). Three males and 3 females were injected for 7 days, 5 males and 5 females for 42 days, excepting Sundays. Five control males and 6 females received no injections.

Histochemical methods. In experiments 1 and 2, the paired adrenals were removed and weighed and the portion of the trachea bearing the thyroids was removed. In experiment 3, the thyroids were dissected free and weighed. In experiment 4 only the adrenals were

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[3] Hibernating hamsters wakens from time to time and drink, void, and then return to the hibernating condition (Lyman and Leduc, 1953). Days of hibernation indicates the period from the day the animal entered hibernation to the day it was killed and discounts the short periods when the animal was awake.

[4] Leeds and Northrup, Model S.

[5] Epinephrine in oil (Adrenalin, Parke, Davis) was generously supplied through Dr. C. A. Bratton, Jr., of the Research Department, Parke, Davis and Co., Detroit, Michigan.

removed. The thyroids were fixed in the Zenker-acetic acid mixture and the adrenals were fixed in 10% neutralized formalin.

After a week of fixation, the adrenals were washed and sectioned at 15 μ on the freezing microtome. They were then prepared by a group of methods thought to identify the cells which form and secrete the steroid hormones of the adrenal cortex (Deane, 1951; Deane and Seligman, 1953). Sections from each gland were stained for total lipids for 1 and 7 minutes in sudan black B, one by the Ashbel and Seligman (1949) method for carbonyl groups, another for cholesterol by the Schultz method. One was mounted unstained for study under the fluorescence and birefringence microscopes. Control sections were first extracted for $\frac{1}{2}$ hour with acetone at room temperature.

Paraffin sections of the thyroids were cut at 5 μ and stained progressively in a dilute mixture of eosin and methylene blue at pH 5.13 for 24 hours (Fawcett and Deane, 1951). These preparations were studied for a decline in colloid basophilia and an increase in thyroid cell height as indices of augmented thyroid activity (Dempsey and Singer, 1946). In experiment 2, calculations of thyroid cell heights on three animals from each group were made according to the method of Starr *et al.* (1939).

RESULTS

General Observations

Experiment 1. Table 1 presents the results for Experiment 1. The body temperature of a number of female hamsters maintained in the stock room was found to average 36.3° C. Body temperature was somewhat lower in animals exposed to the cold (32–34° C.) and had dropped profoundly in hibernators (6–10° C.).

Adrenal weight varied considerably in both controls and in non-hibernators in the cold room, but the averages were similar. Thus, for the warm room controls, the adrenals averaged 16.8 mg.; the adrenal weight relative to final body weight was 16.6 mg.%. For the cold room controls, the adrenals averaged 17.1 mg.; the relative weight was 16.8 mg.%. After hibernation of 2 or 3 weeks, adrenals were consistently small, although before this time adrenal size varied as greatly as in the two control groups. The adrenals from hibernating animals averaged 14.5 mg.; the relative weight was 17.3 mg.%.

Experiment 2. In the preliminary experiment, the rats made no attempt to build nests of the bedding material, while the hamsters, as usual, constructed nests at once. The hamsters tended to lose a few grams on exposure to cold, and there was a slight decline in absolute adrenal weight but no change in relative weight. Their body temperatures, which averaged 36.1° C. (SD $\pm$0.6) at the start of the experiment, dropped about 1° C. on the second day and remained significantly lower ($P = <.01$) for the duration of the experiment (18 days), with considerable daily variation even in the same individual.

The rats gained weight in the cold. Their body temperature, which averaged 36.4° C. (SD $\pm$0.5) at the start of the experiment, showed a significant

TABLE 1. DATA FOR EXPERIMENT 1, COMPARING HIBERNATING FEMALE HAMSTERS WITH CONTROLS IN THE STOCK ROOM AND ANIMALS AWAKE IN THE COLD ROOM

Month animal killed	Day of exper.	Days hibern.	Initial wt., g.	Final wt. g.	Adrenals		Body temp., °C.
					Wt., mg.	Prop. wt., mg./100 g.	
Controls in stock room, 18–35° C.							
May	—	—	—	116	15.5	13.4	—
May	—	—	—	116	17.8	15.3	—
May	—	—	—	100	16.8	16.8	—
June	—	—	88	90	—	—	—
July	—	—	93	95	17.0	17.9	—
Sept.	—	—	84	79	19.0	24.1	—
Sept.	—	—	88	114	19.6	17.2	—
Oct.	—	—	—	106	12.1	11.4	—
Non-hibernators in cold room, 5° C. ±2							
May	6	—	115	106	15.0	14.2	34
June	9	—	97	90	—	—	—
May	18	—	120	129	13.0	10.1	33
May	21	—	122	119	16.0	13.4	34
July	31	—	86	84	19.5	23.2	—
Sept.	98	—	95	91	19.0	20.9	33
Sept.	98	—	93	101	17.7	17.5	32
Sept.	108	—	111	107	19.6	18.3	34
Hibernators in cold room, 5° C. ±2							
July	31	1	96	83	17.5	21.2	8
Aug.	71	1	92	89	22.0	24.7	13
Sept.	106	3	95	88	19.0	21.6	12
Sept.	106	3	99	89	11.7	13.1	10
Sept.	106	12	96	91	20.0	22.0	8
Aug.	71	21	93	84	14.0	16.7	8
Oct.	134	26	97	93	12.3	13.2	23*
Sept.	98	45	72	72	10.0	13.9	6
Sept.	98	54	108	85	11.5	13.5	6
Oct.	134	69	85	78	11.8	15.1	8
Oct.	134	81	79	71	12.0	16.9	7
Oct.	134	83	87	78	12.4	15.9	8

* Waking.

drop to 35.4° C. on the second day. However, thereafter the body temperatures averaged significantly higher (37.0° C.; $P = <.01$). No significant increase in adrenal weight occurred.

In the experiment for which histological results are reported, the hamsters tolerated cold well in spite of the more rigorous conditions, with but one death on the 14th day. A high mortality occurred among the rats, a total of six animals dying between 14 and 21 days. Several of the rats which died bit and mangled their tails, and the resultant loss of blood probably hastened their death. The animals that died are omitted from Table 2.

At the beginning of the experiment the temperatures of the hamsters averaged 36.1° C. (SD ±0.4). There was no decline of the average tempera-

TABLE 2. DATA FOR EXPERIMENT 2, COMPARING THE RESPONSES OF MALE HAMSTERS AND MALE RATS EXPOSED TO COLD (5° C. ±2). THE ANIMALS WERE KEPT IN INDIVIDUAL WIRE-BOTTOM CAGES WITH NO BEDDING

Days in cold	No. animals	Initial wt., g.	Final wt., g.	Adrenal wt., mg.	Adrenal prop., mg./100 g.
			Hamsters		
0	5	128 ± 7	129 ± 6	34.0 ± 3.6	26.3 ± 2.0
3	3	121 ± 11	115 ± 10	34.3 ± 7.4	29.5 ± 4.1
8	5	121 ± 31	97 ± 15	23.5 ± 2.0	24.3 ± 3.6
14	6	133 ± 15	100 ± 10	24.4 ± 4.1	24.3 ± 3.7
21	6	143 ± 13	97 ± 8	25.8 ± 4.5	26.6 ± 4.4
			Rats		
0	5	163 ± 8	183 ± 18	38.2 ± 5.1	21.0 ± 3.1
3	3	191 ± 23	188 ± 23	41.9 ± 4.3	22.5 ± 3.9
7	5	183 ± 15	184 ± 9	45.6 ± 7.6	24.7 ± 3.4
13	7	180 ± 20	204 ± 35	52.9 ± 8.9	26.4 ± 4.9
21	6	175 ± 11	206 ± 26	57.6 ± 14.0	28.4 ± 8.1

All weights include standard deviations.

ture on exposure to cold (36.2° C., SD ±0.5). At the start of the experiment the average temperature of the rats was 36.4° C. (SD ±0.8), and in the cold it averaged 36.2° C. (SD ±0.8), with no significant variation from day to day.

The hamsters lost weight in the cold; their adrenals shrank so that there was no change in proportionate weight of the glands (Table 2). The rats that survived gained weight in the cold, and their adrenal glands enlarged. Because of the weight gain, the rise in absolute weights of the adrenals is more striking ($P = <.01$) than the rise in proportional weight ($P = <.02$).

Experiment 3. The average weight of thyroids of adult control hamsters was 7.3 mg. The thyroids of animals receiving thiouracil rose to 13 mg. in 2 weeks, 30 mg. in 4 weeks, and 70 mg. in 2 months. The iodine content of the thyroid of a control was 14.8 mg.%, and that of an animal receiving thiouracil for 2 months was 1.97 mg.%.

Experiment 4. There was no consistent loss of weight in the animals receiving the injections of epinephrine, and there was no apparent difference in the absolute weights of the adrenals (Table 3). The 13.1% increase in the relative weight of the male glands after 42 days was not statistically significant. The 28.9% increase in the adrenal weight of the females was probably significant ($P = <.05$).

Histological Results

Experiment 1. Adrenal cortex. In female hamsters maintained in the stock room (Table 1), the adrenals uniformly displayed a rather narrow cortex. The zona glomerulosa was approximately 3 to 5 cells in thickness;

TABLE 3. DATA FOR EXPERIMENT 4, COMPARING ADRENAL WEIGHTS OF HAMSTERS RECEIVING DAILY INJECTIONS OF EPINEPHRINE (0.4 MG./KG.) WITH CONTROLS RECEIVING NO INJECTIONS

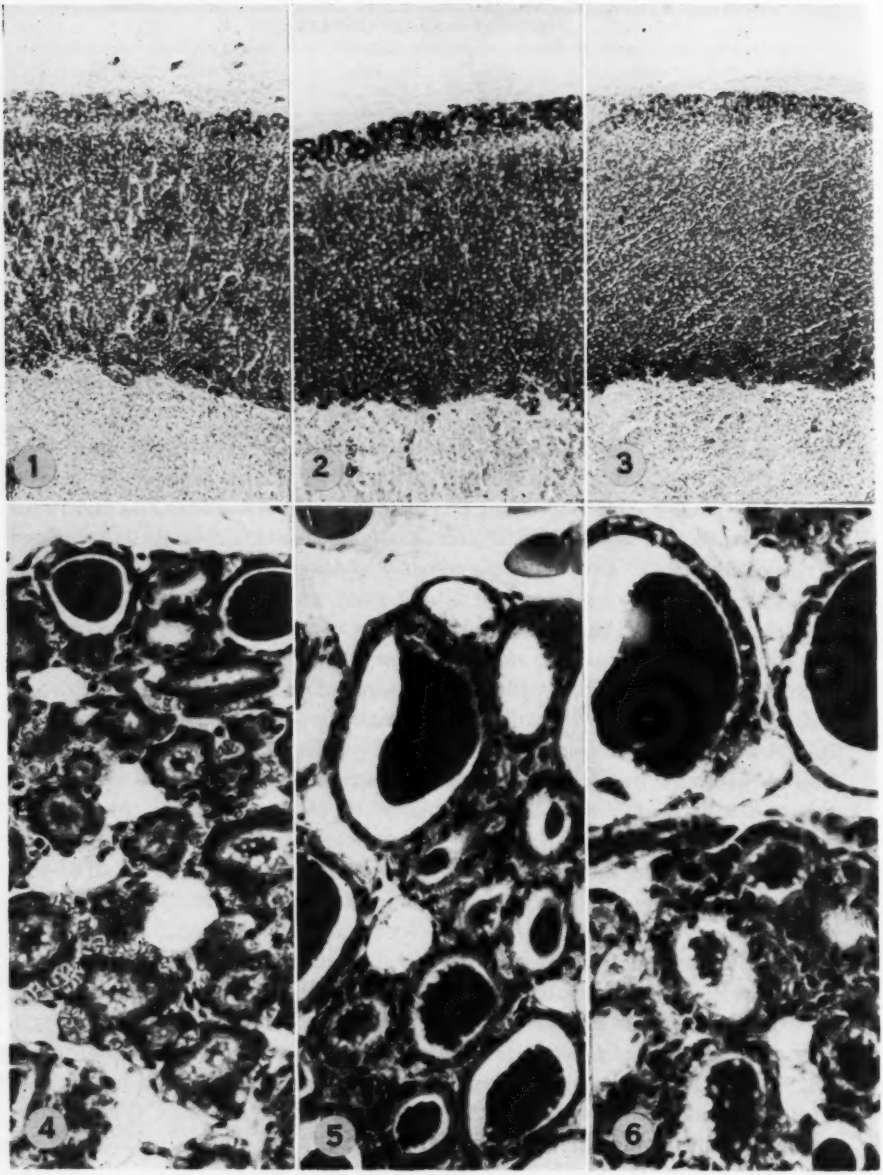
Days injected	No. animals	Initial wt., g.	Final wt., g.	Adrenal wt., mg.	Adrenal prop., mg./100 g.
			Males		
0	5	111 ± 23	110 ± 18	25.5 ± 2.4	23.2 ± 3.4
7	3	130 ± 18	117 ± 17	27.7 ± 1.8	24.1 ± 3.9
42	5	111 ± 16	102 ± 8	26.4 ± 3.5	25.8 ± 2.4
			Females		
0	6	118 ± 13	120 ± 12	17.0 ± 1.8	14.2 ± 1.8
7	3	109 ± 8	108 ± 11	19.3 ± 2.6	18.1 ± 3.2
42	5	94 ± 9	98 ± 9	17.9 ± 3.6	18.3 ± 3.9

All weights include standard deviations.

the cells were small and arranged in small whorls. Beneath the glomerulosa was a transitional zone about 2 cells wide in which the cells were smaller that in either the glomerulosa or the underlying fasciculata. The zona fasciculata was 20 to 25 cells thick; the cells were somewhat larger than those in the glomerulosa. The zona reticularis was about 6 cells in thickness; the cells were shrunken and the cords distorted and compressed. Free lipid droplets staining with sudan black generally occurred only in the glomerulosa, but some specimens showed occasional cells in the reticularis with sudanophilic droplets (Fig. 1). The fasciculata did not contain any lipid droplets, although the cells stained diffusely with the oil-soluble sudan dye. The lipid droplets of the glomerulosa stained positively (blue-purple) by the Ashbel-Seligman method for carbonyl groups; the lipid of the reticularis was generally unstained; the cytoplasm of the cells of the fasciculata stained a diffuse purple. No Schultz cholesterol reaction occurred in any site, nor did any of the droplets contain birefringent crystals. In all of the cells, the cytoplasm emitted a brilliant white fluorescence, not associated with the droplets and not diminished by acetone extraction.

In the female hamsters which did not hibernate and were killed after 6 or 9 days in the cold room, the cortex resembled that of the stock-room controls. In animals exposed to cold for longer periods, the glomerulosal cells enlarged and showed a marked increase in number of lipid droplets (Fig. 2). In the reticularis, more cells appeared shrunken and many had acquired lipid. The fasciculata remained free of fat droplets. There were no changes in droplet histochemistry.

The adrenals of animals that had entered hibernation, resembled those of animals awake in the cold for prolonged periods. The glomerulosa was broader and more fatty than in the warm-room controls and the reticularis



EXPERIMENT 1

was also more fatty (Fig. 3). The histochemical characteristics of the droplets were unchanged.

Thyroid gland. The thyroid gland in the hamster, as in the rat, possesses large follicles with much colloid at the surface of the gland and small follicles with little colloid internally. Occasionally the follicles intermingle with lobules of brown fat. In control animals kept in the stock room, the colloid in the surface follicles was strongly basophilic, while that of the more interior follicles stained only lightly with methylene blue and appeared vacuolated. In all follicles, the cells were cuboidal, but somewhat lower at the periphery than in the center of the gland (Fig. 4). No differences were noted in appearance of glands of animals killed from spring to autumn. In some of the animals exposed to cold and in some which had hibernated, the thyroids resembled the normal glands. In others, there was an increased accumulation of basophilic colloid and flattening of the epithelium (Figs. 5 and 6).

Experiment 2. Adrenal gland, hamster. The adrenal cortex of the normal male hamster is wider than that of the normal female (Peczenik, 1944), but it did not differ in histochemical characteristics (Fig. 8). On exposure of the animals to cold for 3 to 21 days, the cortex appeared somewhat narrower. There was, however, a conspicuous thickening of the glomerulosa and an increase in its lipid content (Fig. 10). In most specimens, no other lipid appeared except in occasional juxtamedullary cells. In a few speci-

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FIGS. 1 to 3, frozen sections of adrenals of female hamsters stained with sudan black B; yellow and orange filters; ×100.

FIG. 1. Hamster kept in stock room, killed in September. All of the cortical cells except those in the subglomerular transitional zone are stained diffusely by the fat-soluble dye; free fat droplets occur in some of the glomerulosal cells and in occasional juxtamedullary cells.

FIG. 2. Hamster in the cold room 98 days, killed in September. The cortex is not enlarged, but the glomerulosa is broader and contains a great deal of fat; more juxtamedullary cells are fatty than in the control.

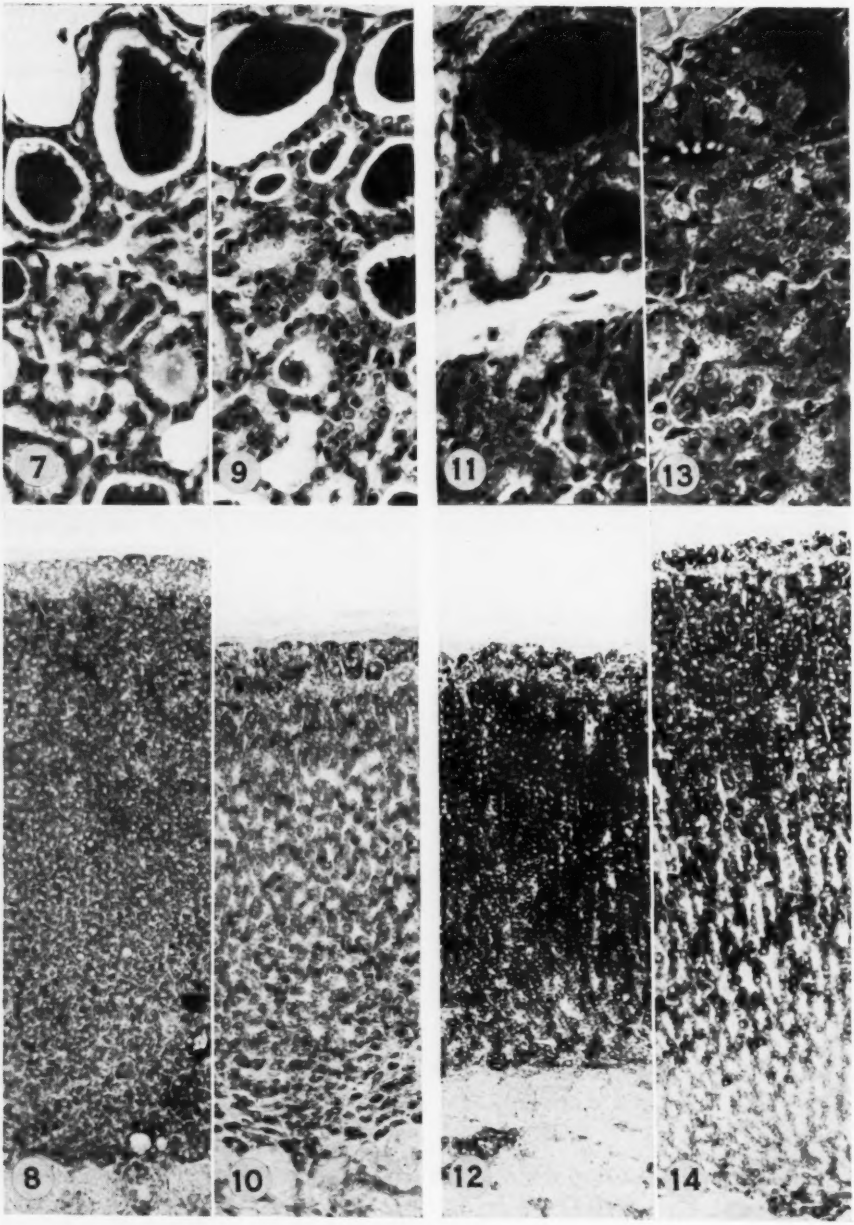
FIG. 3. Hamster in the cold room 134 days, hibernating 81 days, killed in October The glomerulosa is broader and fattier than normal, as is the juxtamedullary zone.

FIGS. 4 to 6, paraffin sections of thyroids of female hamsters, stained with eosin and methylene blue; yellow and orange filters; ×300.

FIG. 4. Hamster in stock room, killed in June The peripheral follicles are large, possess intensely basophilic colloid and a low epithelium. The internal follicles are smaller, have less basophilic, more vacuoluted colloid and cuboidal epithelium.

FIG. 5. Hamster in the cold room 98 days, killed in September. The colloid is denser and the epithelium lower than in the control.

FIG. 6. Hamster in cold room 98 days, hibernating 54 days, killed in September. This thyroid also appears less active than that of the control.



EXPERIMENT 2

TABLE 4. EXPERIMENT 2: AVERAGE HEIGHTS OF THYROID CELLS IN μ, FOR 3 ANIMALS IN EACH GROUP

Days in cold	Rats	Hamsters
0	8.4± .9	7.1±0.4
3	10.4±1.8	8.2±0.6
7	9.0± .9	6.8±0.7
14	10.6± .7	7.9±0.5
21	11.5±1.1	8.1±1.5

Heights include standard deviations.

mens exposed to cold for 8 to 21 days, however, considerable numbers of minute lipid droplets made their appearance in the inner half of the cortex.

Adrenal gland, rat. The adrenal glands of a control rat and of a rat exposed to the cold for 21 days are shown in Figures 12 and 14. As has been reported repeatedly, prolonged cold stress induced an enlargement of the zona fasciculata, with a depletion of lipid from the innermost portion of the cortex. The lipid droplets of the normal rat's adrenal gland were reactive to the Ashbel-Seligman test and the Schultz test, were fluorescent and contained birefringent crystals. In the animals exposed to cold, the lipids of the glomerulosa appeared unchanged but the birefringence and Schultz reaction generally declined in the fasciculata.

Thyroid gland, hamster. The thyroid glands of the normal male hamsters resembled those of the females (Fig. 7). With exposure to cold, the glands did not appear enlarged in section. There was no significant alteration in the heights of the follicular cells (Table 4, and Fig. 9). In some instances, the proportion of follicles containing basophilic colloid appeared to decrease.

Thyroid gland, rat. In section, the thyroid glands of the rats exposed to cold appeared larger than those of the control animals. Moreover, the cells became significantly taller (Table 4, and Figs. 11, 13). Mitoses in the epithelial cells were noted; the number of follicles containing strongly basophilic colloid declined; and vacuolation of the colloid and intracellular droplets staining like colloid increased.

Experiment 3. Histologically, the thyroids of hamsters receiving thiouracil exhibited marked hyperplasia and hypertrophy of the follicular epithelium, with progressive loss of colloid. In the animals treated for 1 or 2

←

Sections prepared and photographed as for Figs. 1 to 6. Thyroids ×300; adrenals ×100.

FIGS. 7 and 8. Thyroid and adrenal cortex of control male hamster.

FIGS. 9 and 10. Thyroid and adrenal cortex of male hamster exposed to cold 21 days.

FIGS. 11 and 12. Thyroid and adrenal cortex of control male rat.

FIGS. 13. and 14. Thyroid and adrenal cortex of male rat exposed to cold 21 days.

months, the follicular walls were markedly invaginated, often producing, in section, the appearance of intrafollicular follicles described by Harris *et al.* (1946) and Kroon (1951).

Experiment 4. Although there appeared to be some increase in the width of the adrenal cortex in the animals injected with epinephrine, there was no alteration in histochemical appearance.

DISCUSSION

Endocrines and hibernation. Thyroid hypertrophy on exposures to cold has been described for a number of mammalian and avian species (Mills, 1918; Spöttel, 1929; Watzka, 1934; Starr and Roskelley, 1940; Bernstein, 1941; Brolin, 1945), and hypertrophy of the adrenal cortex has been recorded for various rodents (Selye, 1937, 1946; Hemphill and Reiss, 1947; Dugal and Thérien, 1949). The results of the present experiments confirm the suggestion that, in a hibernator, exposure to a temperature of 5° C. does not constitute a stimulus to the pituitary to secrete increased amounts of adrenotropin or thyrotropin, since neither the adrenal cortex nor the thyroid become hypertrophied.

Lack of any histological signs of stimulation by cold of the pituitary, thyroid and adrenal cortex has been repeatedly described for other hibernators as well (see introduction). Adler (1926) and Kayser (1950, 1953) have concluded that unresponsivenesss of these glands is responsible for hibernation. The point which seems more important to us, however, is that hamsters often maintain their body temperatures in the cold at normal or only slightly subnormal levels for months despite this lack of endocrine response. Thus, while indeed the ability to hibernate may depend in part on the unresponsiveness of the pituitary and its target organs to cold, the more remarkable fact seems to be that increased activity of the endocrine glands is not at all necessary for maintaining a high metabolic rate in a cold environment.

Skepticism concerning any direct association between endocrine activity and the ability to hibernate has been expressed by several authors (Mann, 1916; Rasmussen, 1921; Johnson and Hanawalts, 1930). On the other hand, Kayser and Aron (1950), Kayser (1950, 1953) and many others consider that, in hibernating species, an involution of the endocrines occurs in the autumn which then permits the animals to become dormant when exposed to cold. However, the present experiments, like those on 13-lined ground squirrels by Zalesky and Wells (1940), were conducted on hamsters which entered hibernation in the spring and summer in the laboratory. Hence it seems unlikely that any peculiarity of the endocrines in autumn is responsible for hibernation.

In many hibernating species that live in the wild, there appears to be

some evidence of hyperactivity of the adrenals and thyroids in the early spring associated with seasonal sexual activity (e.g., 13-lined ground squirrel, Zalesky, 1934, 1935; Foster, 1934; European hamster and dormice, Kayser and Aron, 1950; marmot, Lachiver, 1952). In the Syrian hamster, under laboratory conditions, however, sexual activity is not seasonal, although there is some decline in fertility during the winter months, and there appears to be no seasonal alteration of the endocrine glands. Correspondingly, the Syrian hamster does not evince the marked fluctuations in the size of the reticularis described by Zalesky (1934) and by Kayser and Aron. Appearance of lipid droplets in the reticularis and in the inner fasciculata during shrinkage of the adrenal described here when hamsters were exposed to cold, or when hamsters were treated with estrogen (Alpert, 1950), probably signifies some sort of regressive change. The development of vacuolated cells (lipid-filled?) in this zone when the adrenal is enlarged during the height of reproductive activity (Zalesky, 1934; Kayser and Aron, 1950) is probably of different significance, though we have no interpretation to offer at this time.

All of the hibernating rodents apparently have adrenals which are free of discrete fat droplets except in the zona glomerulosa (Kolmer, 1918; Kohno, 1925; Bourne, 1951). This characteristic is probably not associated with the ability to hibernate, since it also occurs in ruminants and certain nonhibernating insectivores (Bourne, 1951; Nicander, 1952; Verne and Herbert, 1951). Verne and Herbert suggest that the hibernators and ruminants differ from other mammals in that hormone precursors are not stored in the adrenals. Whether this difference indicates less adrenal activity or less dependence on the adrenal cortex in response to stress has not been investigated. It may be germane that hibernating species live longer in the cold after adrenalectomy than do animals which are not hibernators (Mann, 1916; Britton, 1930).

Endocrines and stimuli other than cold. Typical hypertrophy and hyperplasia of the thyroid and decline in thyroid iodine when thiouracil is administered shows that the hamster has a basic requirement for thyroid hormone (see also Harris *et al.*, 1946; Kroon, 1951). However, exposure to 5° C. apparently does not induce an increased output of thyrotropin. In species that show thyroid hypertrophy when exposed to cold, it now appears likely that the hyperactivity follows an increased utilization of the thyroid hormone, rather than being primarily responsible for the increased metabolism (Rand *et al.*, 1952). In the hamster, apparently the increased metabolism in the cold causes no increased consumption of thyroid hormone and hence no stimulation for thyrotropin output.

The adrenal cortex in hamsters has been shown to enlarge somewhat when the animals receive injections of formalin, adrenotropin (Alpert,

1950), or epinephrine (present experiment), but the response is much less marked than in rats or guinea pigs (Chan, 1950). The principal adjustment of the pituitary-adrenal system in the hamster and other hibernators appears to be to gonadal stimuli, and, unlike the situation in the majority of mammals, the hibernating species seem to be unique in that (a) the adrenals are larger in males than in females (Zalesky, 1934; Peczénik, 1944), and (b) androgens rather than estrogens induce adrenal enlargement (Peczenik, 1944; Keyes, 1949). An exception to this generalization is the prairie dog (*Cynomys*). In this species, the adrenal cortex is larger in the female than in the male and may be stimulated by estrogen (Anthony, 1953), but animals will apparently hibernate under proper conditions (Anthony, personal communication).

The response of the zona glomerulosa. One peculiarity observed consistently in hamsters exposed to the cold, even if hibernating, was that the glomerulosa was broader and contained more lipid than in controls. It seems possible that this enlargement signified a stimulation resulting from disturbed electrolyte balance (Deane, Shaw and Greep, 1948; Bacchus, 1950; Eichner, 1953). In this connection, it is worthy of note that casts frequently occur in the kidneys of animals in the cold.

Control of body temperature. The thyroids and adrenals of rats in the cold with bedding gave only slight indications of increased activity, whereas rats deprived of bedding showed increased activity of both glands. In either situation, the animals maintained their normal body temperature. It can be concluded that a temperature of 5° C. *per se* is close to the upper limit of cold which will produce stress in rats of the strain used.

Unlike the rats, the hamsters supplied with bedding showed a significantly lower body temperature during exposure to cold, and there was considerable daily and individual variation in body temperature. Kayser (1939) has observed fluctuations of body temperature in other species of hibernators and has concluded that such imperfect homeothermy is due to an insufficient thermoregulatory apparatus. However, if hamsters were denied bedding when exposed to cold the average body temperature remained normal and fluctuated only slightly. This suggests that when the animals are subjected to greater stress, they show as good thermoregulation as non-hibernators.

Whether different reactions of the endocrines in rats and hamsters are simply a matter of threshold to cold (with rats having the threshold at a higher temperature) cannot be proved by the present experiments. It remains possible that subzero temperatures could produce marked thyroid and adrenal activity in hamsters. On the other hand, we have found that 5° C. and plenty of bedding result in the maximum incidence of hibernation, and the present experiments show that there is no adrenal or thyroid

activation under these circumstances. Certainly the rapid drop in metabolism and body temperature when entering hibernation (Lyman, 1948) emphasizes that the transition from the awake condition of the hibernating state is passive, and the lack of endocrine reaction to temperatres of 5° C. sets the stage for hibernation as soon as the hamster is exposed to cold.

SUMMARY AND CONCLUSIONS

A widely accepted theory of hibernation is that the endocrine glands which are intimately connected with body temperature involute in the autumn, and this involution is a causative factor in the onset of hibernation. Such an involution of thyroid and adrenal as environmental temperatures become colder is opposite from the usual effect found in animals which do not hibernate. In order to clarify this difference, the histophysiological responses to cold of the thyroids and adrenals were studied in the Syrian hamster and the albino rat, exposed to identical stress of 5° C. Suitable controls were kept at 23° C. Body temperatures were obtained from both groups.

When compared with controls, non-hibernating hamsters exposed to the cold, as well as animals that had been in the hibernating state for 1 to 83 days, showed no enlargement of the adrenals, nor any histophysiological evidence of hyperactivity of either the zona fasciculata of the adrenal cortex or of the thyroid. However, enlargement and increased lipid content of the zona glomerulosa of the adrenal developed, and free lipid made its appearance in the shrunken zona reticularis. In contrast, rats exposed to identical cold environment showed histophysiological signs of increased activity of both glands. Rats maintained their body temperatures well in the cold, whether or not provided with bedding, whereas hamsters displayed more stable body temperatures when deprived of bedding.

Experimental treatments other than cold, however, did induce typical endocrine responses in hamsters. Thiouracil administration caused enlargement of the thyroids; injections of epinephrine for 7 or 42 days produced some adrenal enlargement.

Hibernators contrast with non-hibernators in that neither the pituitary-adrenal nor pituitary-thyroid axis seems to be stimulated by moderate cold. The adrenals of hibernators seem to alter principally with respect to sexual activity; further, the adrenals of hibernators are remarkably lipid-poor. Whether these peculiarities of the adrenals of hibernators bear any relation to the ability to hibernate remains unsettled.

It is concluded that polyglandular insensitivity of the endocrines to cold is typical of hibernators but is not a causative factor in the process of entering hibernation. In the hamster, the homeothermic state can be maintained with no involvement of thyroid or adrenal cortex at temperatures

which produce hyperactivity of these glands in the rat. This lack of endocrine reaction to cold may be permissive of hibernation, but does not control the onset of the hibernating state.

ADDENDUM

After this paper was in press, an excellent study on the cytology and histochemistry of the adrenal cortex of normal and hypophysectomized hamsters was published by Knigge (1954). Thirty days after hypophysectomy, the adrenals of males weighed 45% of normal, those of females 60%. As in the rat, the zona glomerulosa remained healthy in appearance, whereas the inner zones atrophied. Earlier Agate (1952) found that either enlargement of the glomerulosa (after gonadectomy) or administration of desoxycorticosterone, both in hamsters bearing transplants of mouse sarcoma, resulted in marked edema and salt retention. It thus appears likely that the glomerulosa of the hamster secretes a homone which causes salt retention. Agate also observed that in intact hamsters, the sarcoma transplants induced some enlargement of the inner zones of the cortex, accompanied by the accumulation of lipid droplets; in hypophysectomized animals no such changes occurred. These results indicate that a sufficiently noxious stimulus is capable of activating the pituitary to cause a characteristic hypertrophy of the fasciculata and the appearance of lipid in its cells.

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EFFECTS OF BREEDING ON THE DEVELOPMENT OF OVARIAN, ADRENAL, AND UTERINE LESIONS IN DBA×CE AND RECIPROCAL HYBRID MICE[1]

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IN DBA×CE and reciprocally crossed hybrid mice hyperplasia of the uterus occurs spontaneously in both intact virgin and neonatally ovariectomized females at 6 to 8 months of age and persists throughout life (Christy, Dickie, Atkinson and Woolley, 1951). The uterine lesions include hyperplasia of the endometrial glands, adenomyosis, hyperplasia of the uterine muscle and generalized fibrosis and vascular congestion. These lesions, however, are but one manifestation of a widespread endocrine imbalance involving the anterior lobe of the hypophysis, the adrenal cortex and the ovaries. In the ovariectomized female the uterine hyperplasia develops as the result of hyperestrinism due to aberrant hormone production by the hyperplastic and neoplastic adrenal cortical lesions which appear following gonadectomy. In the intact virgin, on the other hand, the hyperestrinism is mediated through the ovaries (Atkinson and Dickie, 1953). In both the castrate and intact virgin considerable amounts of progesterone and androgen are also produced.

In spite of the development of hyperovarianism with consequent morphologic alterations in the uteri of virgin females after 6 months of age, females mated shortly after puberty breed vigorously and continue to produce and raise litters until they are well over 12 months of age. Since earlier studies of these and other strains of mice exhibiting similar abnormalities have been limited to the examination of intact virgin and prepuberally ovariectomized animals (Fekete *et al.*, 1941; Woolley and Little 1945 a, b and c: Frantz *et al.*, 1947; Smith, 1948; Dickie and Woolley, 1949), the present study was undertaken to ascertain the effects of breed-

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ing on the development of the ovarian, adrenal and uterine lesions which occur spontaneously in the unbred female.

MATERIALS AND METHODS

The mice used in the present investigation were the F_1 offspring of reciprocally crossed DBA/2Wy and CE/Wy strains. Twenty females, 16 to 24 months of age, were selected from the Jackson Laboratory colony. Ten had been separated from males since weaning and 10 had been housed with males continuously from the age of 2 to 4 months. The latter females had produced and raised from 2 to 10 litters apiece (Table 1).

The selected animals were sacrificed and tissue specimens from the uteri, vaginas and submaxillary glands were taken. These, together with the entire adrenal glands and ovaries, were fixed in Vandergrift's fluid and were subsequently embedded in paraffin. Cross sections were cut 6 micra in thickness and were stained with hematoxylin and eosin for microscopic study.

The extent of the uterine lesions was recorded on the basis of the morphologic changes in the endometrial glands as previously described (Atkinson and Dickie, 1953). If only an occasional gland exhibited cystic or adenomatous change, the lesion was classified as being "slight." If a substantial minority of glands were hyperplastic or if early adenomyosis were found, the lesion was designated "moderate." When extensive glandular hyperplasia and/or adenomyosis were present, the lesion was recorded as "marked."

The endogenous steroid sex hormone activity at the time of sacrifice of the animals was estimated from the cytology of the epithelium of the vaginas and from that lining the terminal ducts of the submaxillary glands using the criteria described in our previous papers cited above. In brief, the patterns of vaginal epithelium described by Freud (1938) were adopted: Type I indicates the virtual absence of circulating estrogen; Type II is characteristic of relatively weak estrogenic activity; Type III indicates strong combined stimulation by estrogen and progesterone and/or androgen; Type IV indicates strong stimulation by estrogen alone. In the submaxillary gland the terminal ducts in female and castrated male mice are lined by a low columnar epithelium with centrally located spherical nuclei. Moderate androgenic stimulation induces heightening of the epithelium with migration of the nuclei to the basal zone. More intense androgenic activity, comparable to that in intact adult males, is accompanied by further heightening of the epithelium and by flattening of the nuclei (Lacassagne, 1940).

OBSERVATIONS

The results are summarized in Table 1.

Virgins

The morphologic findings in the virgin mice agree with those previously found in these hybrid strains under similar conditions (Christy *et al.*, 1951; Woolley *et al.*, 1952; Atkinson and Dickie, 1953) and are briefly described below.

Ovaries.—The ovaries were grossly enlarged. Microscopic examination revealed that their increased size was due to the presence of large numbers of hyalinized corpora lutea which made up the bulk of the organs (Fig. 1). In 7 of the animals one or more cysts of undetermined origin were present;

TABLE 1. THE EFFECTS OF BREEDING ON THE MORPHOLOGIC MANIFESTATIONS OF THE HYPEROVARIAN SYNDROME IN DBA×CE AND RECIPROCAL HYBRID MICE

AGE AUTOPSIED	BREEDING RECORD (BY MONTHS OF AGE) 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20	OVARIAN LESIONS: CYSTS	OVARIAN LESIONS: HYALINIZED CORPORA LUTEA	EXTENT OF UTERINE LESIONS (OTHER THAN VASCULAR)	TYPE OF VAGINAL CYTOLOGY	TYPE OF SUBMAXILLARY CYTOLOGY	SUBCAPSULAR "A" CELL FORMATION IN ADRENAL CORTEX
16 MOS.	♂ L L L L L	NONE	NONE	NEGLIGIBLE	II	♀	BAND ABSENT, EXTENSIONS INTO Z. FASCICULATA PRESENT
16 MOS.	♂ L L L L L L L L L	NONE	NONE	NEGLIGIBLE	III	♀	INCOMPLETE BAND
17 MOS.	♂ L L	NONE	NONE	SLIGHT	II	INTERMEDIATE	SCATTERED SMALL NODULES
17 MOS.	♂ L L L L L L	NONE	NONE	NEGLIGIBLE	II	♀	INCOMPLETE BAND
18 MOS.	♂ L L L L L L L L	NONE	NONE	NEGLIGIBLE	II	INTERMEDIATE	BAND ABSENT, EXTENSIONS INTO Z. FASCICULATA PRESENT
18 MOS.	♂ L L L L L L L L	NONE	NONE	NEGLIGIBLE	IV	INTERMEDIATE	BAND ABSENT
18 MOS.	♂ L L L L L L L L L	NONE	NUMEROUS	NEGLIGIBLE	III	♀	SCATTERED SMALL NODULES
20 MOS.	♂ L L L L L L L L L	2	NONE	NEGLIGIBLE	IV	♀	BAND ABSENT, EXTENSIONS INTO Z. FASCICULATA PRESENT
20 MOS.	♂ L L L L L L L L	NONE	NUMEROUS	SLIGHT	II	♀	BAND ABSENT, EXTENSIONS INTO Z. FASCICULATA PRESENT
21 MOS.	♂ L L L L L L L L	NONE	NONE	SLIGHT	II	INTERMEDIATE	BAND ABSENT, EXTENSIONS INTO Z. FASCICULATA PRESENT
18 MOS.	VIRGIN	NONE	NUMEROUS	MARKED	IV	♀	COMPLETE BAND
18 MOS.	"	SEVERAL	NUMEROUS	MARKED	III	INTERMEDIATE	INCOMPLETE BAND
18 MOS.	"	NONE	NUMEROUS	SLIGHT	II	♀	INCOMPLETE BAND
18 MOS.	"	1 LARGE	NUMEROUS	MARKED	IV	INTERMEDIATE	INCOMPLETE BAND
19 MOS.	"	1	NUMEROUS	MARKED	IV	INTERMEDIATE	COMPLETE BAND
20 MOS.	"	1 LARGE	NUMEROUS	MARKED	II	INTERMEDIATE	INCOMPLETE BAND WITH EXTENSIONS INTO Z. FASCICULATA
22 MOS.	"	1 (LUTEAL)	NUMEROUS	MARKED	II	INTERMEDIATE	INCOMPLETE BAND WITH EXTENSIONS INTO Z. FASCICULATA
22 MOS.	"	2 SMALL	NUMEROUS	MODERATE	III	INTERMEDIATE	INCOMPLETE BAND WITH EXTENSIONS INTO Z. FASCICULATA
24 MOS.	"	1	NUMEROUS	MODERATE	IV	♀	INCOMPLETE BAND
24 MOS.	"	NONE	NUMEROUS	MODERATE	II	♀	INCOMPLETE BAND WITH EXTENSIONS INTO Z. FASCICULATA

♂—age when placed with a male.
L—month of age in which a litter was born.
II—non-cornified stratified squamous vaginal epithelium.
III—mucified vaginal epithelium.
IV—cornified stratified squamous epithelium.

in 6 of these animals the hyalinized corpora lutea were partially calcified. The ovaries from all the mice contained some normal and atretic follicles, but these were very few in number in the animals 19 months and older. A few normal corpora lutea were also found in all instances.

Adrenals.—The principal feature of interest in the adrenal cortex was the consistent proliferation of subcapsular cells (Fig. 2). These are small cells with compact nuclei, slightly basophilic cytoplasm and indistinct cell membranes. They have been termed "A" cells by Woolley and Little (1945 a). Their origin and function are not clear. In the present animals they were found to form wide bands under the capsules of the adrenals and to send columnar extensions into the underlying zona fasciculata.

Uteri.—In general the uteri were grossly enlarged and hyperemic. Microscopically, the endometrial glands showed extensive hyperplasia with frequent extension into the myometrium (Fig. 5). The uterine muscle was hyperplastic and the myometrium edematous. The small blood vessels throughout the uterus were markedly dilated, particularly in the connective tissue between the muscular layers of the myometrium and in the peripheral portion of the endometrium.

Vaginas.—In all mice the vaginal epithelium showed cytologic evidence of hormonal stimulation. Cornification (Type IV) was present in 4 animals and mucification (Type III) in 2.

Submaxillary Glands.—The epithelium of the terminal ducts in 6 of the mice showed moderate heightening and basal migration of the nuclei which, however, remained spherical in shape. In none of these animals were the cytologic characteristics as marked as those present in normal intact males.

Breeders

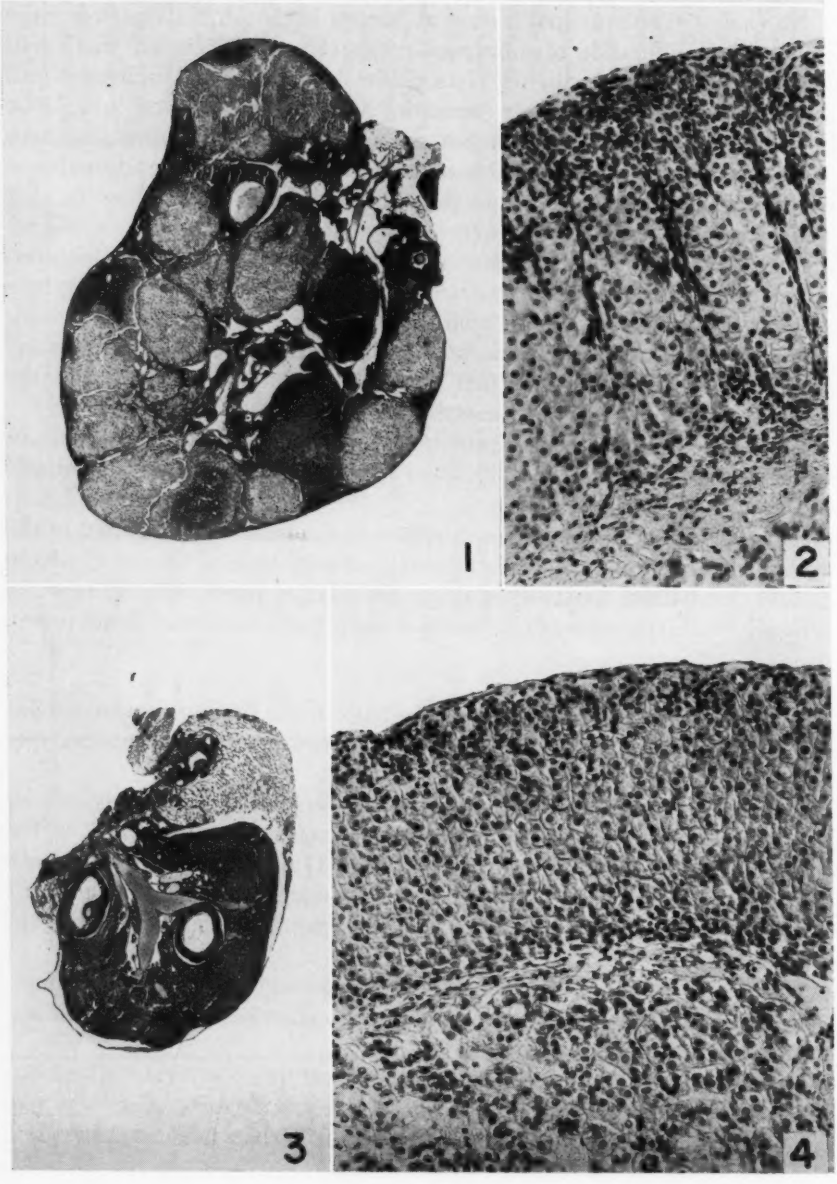
In some respects the morphologic findings in the bred mice were similar to those present in the virgins, but in other respects they were strikingly different.

Ovaries.—In general the ovaries of the breeders were considerably smaller than those of the virgins. No pathologic changes were found in 7 of the animals (Fig. 3). As would be expected, after 17 months of age there was a marked reduction in the numbers of developing and atretic follicles. In 1 of the remaining 3 animals, two cysts were present in otherwise normal appearing ovaries.

The ovaries of 2 of the breeders resembled those of the virgin animals described above, i.e. the organs were grossly enlarged and contained numerous hyalinized corpora lutea.

Adrenals.—The adrenals of the bred mice exhibited several striking differences from those of the virgins. The cortex was narrower and there was much less proliferation of subcapsular "A" cells than in the virgins (Fig.

PLATE I



4). These cells did not form a continuous band between the capsule and zona glomerulosa, but were more scattered. There was also considerably less tendency to project into the zona fasciculata.

A further difference between the adrenals in the two groups was quite surprising. In the breeders a fairly heavy leucocytic infiltration was consistently present in the zona reticularis and in two adrenals evidence of extra-medullary myelopoiesis was also observed. The adrenals of the virgins did not exhibit this leucocytic infiltration.

Uteri.—In general the uteri were smaller grossly than those of virgins, but showed a similar hyperemia. Microscopically, there was almost a complete absence of pathologic change in the endometrial glands, only two animals showing occasional cysts and a third showing a small area of adenomyosis. The myometrium, on the other hand, was enlarged and edematous (Fig. 6). The vascular congestion throughout these uteri was relatively more striking than in the uteri of the virgins due to the absence of cystic and adenomyotic glands. Considerable numbers of pigment-containing macrophages were also present throughout the uteri, particularly in the mesometrial connective tissue.

Vaginas.—As in the virgin mice, the vaginal epithelium showed some degree of hormonal stimulation in every instance. Cornification (Type IV) was found in two animals and mucification (Type III) in two others.

Submaxillary glands.—There was moderate heightening of the terminal duct epithelium with basal migration of spherical nuclei in 4 mice. No evidence of androgenic stimulation was present in the remaining animals.

DISCUSSION

It is readily apparent from the observations recorded above that breeding markedly suppresses the development of several morphologic manifestations of the endocrine imbalance which arises spontaneously in virgin female mice of these hybrid strains. The biologic mechanisms by which breeding produces these suppression effects can only be speculated upon,

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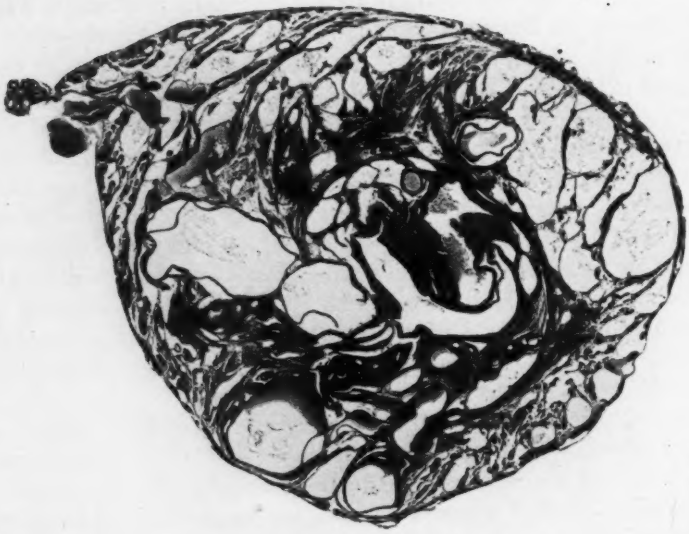
FIG. 1. Ovary from a 20 month old virgin hybrid mouse showing the presence of numerous lightly stained hyalinized corpora lutea. (×18).

FIG. 2. Adrenal cortex from the same animal as in Fig. 1 illustrating the marked proliferation of small darkly stained "A" cells forming a subcapsular band and showing the columnar extensions of these cells into the underlying zona fasciculata. (×160).

FIG. 3. Ovary from a 20 month old hybrid mouse which had produced and raised 8 litters. Compare with Fig. 1, noting particularly the absence of hyalinized corpora lutea. (×18).

FIG. 4. Adrenal cortex from the same animal as in Fig. 3. Compare with Fig. 2, noting particularly the great reduction in the proliferation of subcapsular "A" cells. (×160).

PLATE II



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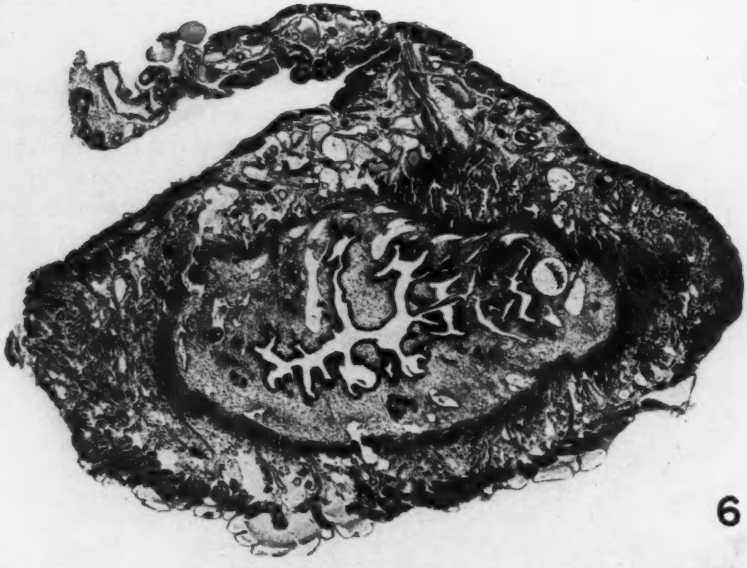

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since our knowledge of the pathogenesis of the hypergonadal syndrome is at best imperfectly understood. The distinctive lesion in the ovaries of the virgin mice is the accumulation of large numbers of hyalinized and calcified corpora lutea. It is probable that these accumulate in the same manner as that previously described in virgin females of the parent DBA strain (Fekete, 1946). In the DBA each recent set of corpora lutea fails to regress during the several immediately succeeding estrous cycles as normally occurs in most strains of mice. Instead, the corpora lutea in the DBA strain tend to persist for as many as 6 or 7 cycles so that large numbers accumulate in the ovaries. The absence of accumulated corpora lutea in the majority of the present bred hybrid mice might tentatively be explained as the result of the reduction in the number of generations of corpora lutea due to the succession of pregnancies and lactations and also as the result of the regression of previous generations of corpora lutea during pregnancy itself. More specifically, in virgin mice succeeding sets of corpora lutea are produced at intervals of less than one week (i.e., the duration of the estrous cycle), whereas in bred animals succeeding sets of corpora lutea are produced at intervals of approximately 4 weeks (i.e., the duration of pregnancy plus the period of delay of implantation during early lactation). The failure of corpora lutea to accumulate in the ovaries of the bred animals between the birth of their last litter and the time of their sacrifice (an average of $3\frac{1}{2}$ months) is probably related to the declining reproductive activity of the animals due to advanced age. The presence of numerous hyalinized corpora lutea in the ovaries of two of the animals bred repeatedly remains unexplained, unless it is assumed that in these animals the ovaries continued their cyclic activity after the birth of the last litters.

Our knowledge of the physiologic potentialities of the various components of the pathologic ovaries in these mice is even more limited than that of the pathogenesis of the ovarian lesions themselves. Our previous studies, however, have established several pertinent facts concerning the endocrine aspects of the syndrome. From the histologic and cytologic characteristics of the uterus and vagina in the aged virgin it is evident that there is an excessive production of steroid sex hormones, either quantitatively or temporally, and/or excessive tissue sensitivity to hormonal stimulation (Christy *et al.*, 1951). From extirpation experiments in virgins it is evident that the ovaries, not the adrenals, are the major source of the steroid sex

FIG. 5. Uterus from the same animal as in Fig. 1 showing the marked cystic glandular hyperplasia, adenomyosis and vascular congestion. (×18).

FIG. 6. Uterus from the same animal as in Fig. 3. Compare with Fig. 5, noting the similar vascular congestion but absence of glandular lesions. (×18).

hormones produced in these animals (Atkinson and Dickie, 1953). The present observation that lesions of the endometrial glands fail to develop in bred animals is correlated with the absence of ovarian lesions in the majority of cases. The two bred animals with morphologically abnormal ovaries and normal uterine glands would appear to indicate that these phenomena are physiologically unrelated. However if it may be assumed that in these instances the corpora lutea had accumulated since the weaning of the last litters (1 and 3 months respectively), the time interval for uterine lesions to have developed is too short (Christy *et al.*, 1951). The occurrence of vaginal mucification (Type III) and appreciable androgenic stimulation of the submaxillary glands in several of the bred mice, on the other hand, minimize retention of the corpora lutea as a major etiologic factor in the hypergonadal syndrome. It is also of interest to note that vascular congestion and myometrial hypertrophy occurred in all the bred animals whether or not pathologic changes appeared in the ovaries. From these varied observations it is apparent that at present we have insufficient data from which to draw any definitive conclusions concerning the sites of hormone production within the pathologic ovaries or concerning the exact nature of the endocrine imbalance itself.

The partial suppression of "A" cell proliferation in the adrenal cortices of the bred animals is of particular interest in view of the recent studies of Woolley, Dickie and Little (1952). These authors have observed that in DBA×CE and reciprocal hybrid virgin female mice "A" cells begin to accumulate in the subcapsular cortex at about 3 months of age and continue to accumulate with advancing age as described in the present animals. In mice ovariectomized at birth, on the other hand, foci of hypertrophic ("B") and neoplastic cells develop in association with the "A" cells. The proliferation of "A" cells is genetically determined; the physiologic mechanisms responsible for their development and for the subsequent neoplastic alterations in the castrate have not been clearly demonstrated. Since the present observations have shown that repeated breeding suppresses "A" cell growth, it seems probable that the ovaries are implicated either through direct hormonal effects on the adrenal cortex or indirectly through the anterior pituitary gland.

SUMMARY

After 6 to 8 months of age virgin female DBA×CE and reciprocally crossed hybrid mice spontaneously develop hyperovarianism in association with delay in the regression of corpora lutea, proliferation of "A" cells in the adrenal cortex and marked glandular and muscular hyperplasia of the uterus. Females mated at 2 to 4 months of age, however, breed vigorously until well after 12 months of age. The present study was made to determine

the effects of breeding on the hyperovarian syndrome. Microscopic study of the ovaries, adrenals and uteri of females 16 to 24 months of age which had produced and raised 2 to 10 litters apiece revealed that the morphologic manifestations of endocrine imbalance were either partially or completely suppressed. The possible physiologic mechanisms by which pregnancy and lactation might produce these suppression effects have been discussed.

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STEROID TRANSFORMATIONS BY CORPUS LUTEUM TISSUE[1]

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STUDIES have been initiated in this laboratory to investigate the steroid metabolic activity of ovarian tissue. A preliminary note of certain findings, i.e., the demonstration of 6β-hydroxylase and 20-hydrogenase activities, appeared earlier this year (Hayano *et al.*, 1953). Following the incubation of progesterone and desoxycorticosterone (DOC) with beef corpus luteum homogenates, the isolation and identification of Δ^4-pregnene-20β-ol-3-one from the former, and Δ^4-pregnene-20β,21-diol-3-one, and Δ^4-pregnene-6β,21-diol-3,20-dione from the latter steroid, has now been completed.

The first isolation of a 6β-hydroxylated steroid from mammalian sources was reported by Haines (1952) who obtained Δ^4-pregnene-6β,21-diol-3,20-dione after incubation of a hog adrenal homogenate with DOC. Subsequent isolations have been reported by Zaffaroni[2] after a similar incubation using a beef adrenal homogenate, and by Axelrod (in press) after perfusion of a rat liver with this precursor. Other 6β-hydroxylated steroids which have been isolated are: 6β-hydroxyprogesterone by Levy and Kushinsky[3] after perfusion of progesterone through beef adrenals, 6β-hydroxycortisol[4] by Burstein[5] from guinea pig urine after the animals were treated with cortisol, and 6β-hydroxy-Δ^4-androstene-3,17-dione by Meyer and Hayano[6] after incubation of Δ^4-androstene-3,17-dione with an adrenal

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[2] Zaffaroni, A., personal communication.

[3] Levy, H., and Kushinsky, S., unpublished data.

[4] Cortisol has been suggested as a more convenient name than hydrocortisone for the compound, Δ^4-pregnene-11β,17α,21-triol-3,20-dione (Shoppee, 1953).

[5] Burstein, S., unpublished data.

[6] Meyer, A. S., and Hayano, M., unpublished data.

homogenate preparation. Another biological source is the Mucorales fungus which has been shown to yield 6β-hydroxylated derivatives from a variety of steroid precursors (Murray and Peterson, 1952).

The existence of 20β-hydroxylated C_{21}-steroids in animal tissues has been known for some time (Reichstein and Shoppee, 1943). The *in vitro* reduction of the C-20 ketone has been noted more recently in a number of studies with tissue preparations. Schneider (1952) isolated allopregnane-3β, 20,21-triol after incubating DOC with rat liver slices. The configuration at C-20 was not determined. The bio-hydrogenation of the C-20-keto group has been demonstrated in several other instances. Caspi and co-workers have identified Δ^4-pregnene-17α,20β,21-triol-3,11-dione (1953), allopregnane-3β,17α,20β,21-tetrol-11-one, and Δ^4-pregnene-11β,17α,20β,21-tetrol-3-one after perfusion of rat livers with cortisone; the latter product was found again after perfusions with cortisol.[7] Δ^4-Pregnene-17α,20β,21-triol-3,20-dione has been isolated by Forchielli (1953) after incubation of a rat liver homogenate with Δ^4-pregnene-17α,21-diol-3,20-dione.

METHODS

Tissue Preparations

Beef ovaries were collected immediatedly after slaughter. The corpora lutea were dissected, frozen on dry ice, and kept frozen until just before use. The tissue was used within one or two days. While still partially frozen, the glands were cut into slices, using a razor blade. These slices were homogenized in a Waring blendor with 100 ml. of a solution consisting of 0.1 M sodium fumarate, pH 7.4, 0.01 M $MgCl_2$, and 0.03 M sodium phosphate buffer, pH 7.4, being used per 100 grams of tissue. The homogenate was strained twice through four thicknesses of gauze to remove gross particles.

Incubations

Incubations were carried out at 38° C. in 125 ml. Erlenmyer flasks. 10 grams equivalent of tissue (about 16 ml. of homogenate) were placed in each flask containing 8–10 mg. of steroid in 0.4–0.5 ml. of propylene glycol and incubated for 90 minutes with constant shaking. The gas phase was air. Neutralized adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN), to make a final molarity of 0.002 M each, were added 20 minutes after the start of the incubation.

Extraction

The incubate was poured into 10 volumes of methanol or acetone, the mixture stirred for 3 hours at 6° C., and allowed to stand overnight. The preparation was filtered and the tissue reextracted twice with additional solvent. The combined extracts were distilled *in vacuo* to a 70% organic solvent solution, partitioned against ligroin to remove fats, and the aqueous solvent solution concentrated to an aqueous sludge. This fraction was then extracted with chloroform which was then concentrated to dryness. The oily residue thus obtained was subjected to chromatography on a silica gel column using as eluting media varying proportions of ethyl acetate in benzene. The steroids thus resolved were analyzed by paper chromatography, using the toleune-propylene glycol system

[7] Caspi, E., unpublished data.

CH3
C=O
O
PROGESTERONE
CH3
HOCH
O
Δ4 - PREGNENE -
20β - OL - 3 - ONE
CH2OH
C=O
O
DESOXYCORTICOSTERONE
CH2OH
HOCH
O
Δ4 - PREGNENE -
20β, 21 - DIOL - 3 - ONE
CH2OH
C=O
OH
O
Δ4 - PREGNENE -
6β, 21 - DIOL - 3, 20 - DIONE

of Zaffaroni, Burton, and Keutmann (1950), or the ligroin-propylene glycol modification of Savard (1953). Reagents employed were acidic 2,4-dinitrophenylhydrazine (DNPH for the Δ^4-3-keto group (orange color), and alkaline triphenyltetrazolium chloride (TPTZ) for the α-ketol reducing function (pink co'or).

Physical Constants

All melting points were observed on a Fisher-Johns hot stage and are corrected. Infrared analyses were made on a 12 C Perkin-Elmer infrared spectrometer on solid films.[8]

EXPERIMENTAL

Desoxycorticosterone Incubation

240 mg. of chromatographically pure DOC, m.p. 143.5–145.5°, in 9.6 ml. of propylene glycol were incubated with 300 gm. of corpora lutea and the mixture extracted with methanol and purified as described in the section on methods. Eluates from the silica gel chromatography, analyzed by paper chromatography using the toluene-propylene glycol system, gave the results tabulated in Table 1. The silica gel fractions containing what appeared to be a single transformation product more polar than desoxycorti-

TABLE 1. SILICA GEL CHROMATOGRAPHY OF PROGESTERONE AND DESOXYCORTICOSTERONE INCUBATIONS WITH CORPUS LUTEUM TISSUE

Progesterone incubation		Desoxycorticosterone incubation	
Eluate fractions	Steroids isolated	Eluate fractions	Steroids isolated
12–14% ethyl acetate in benzene	progesterone		
17–33% ethyl acetate in benzene	Δ^4-pregnene-20β-ol-3-one	10–25% ethyl acetate in benzene	DOC
		33% ethyl acetate in benzene—100% ethyl acetate	Δ^4-pregnene-20β, 21-diol-3-one and Δ^4-pregnene-6β, 21-diol-3,20-dione

costerone, and demonstrating both the orange DNPH and the pink TPTZ color reactions, were combined and crystallized in acetone-ether. 15 mg. of a compound, m.p. 162–167°, were obtained, which, when analyzed on paper showed a strong orange DNPH reaction but no color with TPTZ. Recrystallization of this substance in acetone brought the melting point up to 167–168.5°. Infrared analyses of this substance and its acetylated derivative showed that it was clearly different from Δ^4-pregnene-20α,21-diol-3-one (m.p. 167–170°) and its diacetate and identical with Δ^4-pregnene-20β,21-diol-3-one (m.p. 166–168°) and its derivative.

[8] The infrared analyses were carried out by Mr. P. Skogstrom.

From the mother liquors of the above crystallization, 8 mg. of another component, melting between 178° and 187°, were obtained. This material gave positive reactions for the presence of both the α,β-conjugated ketone and α-ketol groupings. On recrystallization from acetone, the melting point was raised to 186–190°. An infrared analysis of this material showed that it was identical with an authentic sample of Δ^4-pregnene-6β,21-diol-3,20-dione (m.p. 190–192°).

Progesterone Incubation

250 mg. of chromatographically pure progesterone (m.p. 131–132°) in 12.5 ml. propylene glycol were incubated with 250 gm. of corpus luteum tissue and the mixture extracted with acetone and purified as described in the section on methods. Eluates from the silica gel column chromatography were analyzed by paper chromatography, using the ligroin-propylene glycol system. The results are presented in Table 1. Only one major transformation product was found which when crystallized from acetone (35 mg.) gave a melting point of 170.5–173°. Determination of a mixed melting point (170–173°) and the comparison of the infrared spectra of this substance and its acetylated derivative with authentic samples of Δ^4-pregnene-20β-ol-3-one (m.p. 170–172°) and its acetate established their identities.

Tissue Blanks

Corpus luteum tissue processed in the manner described showed the presence of only one component in small quantities exhibiting a paper mobility rate identical with that of progesterone.

SUMMARY

The isolation of Δ^4-pregnene-20β-ol-3-one after the incubation of progesterone, and Δ^4-pregnene-20β,21-diol-3-one and Δ^4-pregnene-6β,21-diol-3,20-dione after the incubation of desoxycorticosterone with beef corpus luteum homogenates is described.

Acknowledgments

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COMPARATIVE DISTRIBUTION AND FATE OF I^{131}-LABELED THYROXINE AND TRIIODOTHYRONINE[1]

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GROSS and Leblond (1951 a, b) first demonstrated a substance in tissues (unknown 1) which, on the basis of subsequent studies (Gross and Pitt-Rivers 1953 a) apparently was 3,5,3′L-triiodothyronine. Metabolic effects of this material have been investigated in animals, and in normal and myxedematous humans (Asper, Selenkow and Plamondon, 1953, Gross and Pitt-Rivers, 1953 b, Heming and Holtkamp, 1953, Lerman, 1953, Rall *et al.*, 1953, Rawson *et al.*, 1953, and Starr and Liebhold-Schueck, 1953 a & b.). In the athyreotic individual, L-triiodothyrone has been shown to be from 3.5 to 10 times as calorigenic as L-thyroxine in the first few days of therapy. It can produce an increase in metabolic activity in a remarkably short time, effecting clinical improvement within four to six hours (Asper, Selenkow and Plamondon, 1953) in contrast to the delayed metabolic effect and prolonged action of thyroxine.

Until labeling of thyroxine with I^{131} was accomplished (Joliot *et al.*, 1944) determination of its concentration in tissues of the body was difficult and inaccurate. Since then, several reports have appeared in which distribution of tagged thyroxine was investigated. Gross and Leblond (1947), using large doses of I^{131} labeled thyroxine, first studied its distribution in rats. In 1950, using more physiological doses, they found that thyroxine was removed from plasma at a rapid rate, only 26% of the injected dose remaining at the end of 2 hours. This level gradually dropped to 8% at 24 hours and 1% at 72 hours. At two hours the highest tissue concentration was found in liver. This was also true at 24 hours, although by 72 hours the concentration of the radioactivity had declined to a level little different from that of plasma. Kidney, stomach, muscle and intestine showed relatively small concentrations, except for a high (14%) concentration in the small intestine at two hours.

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[2] U. S. Public Health Service Research Fellow.

Myant and Pochin (1950) found that when I^{131}-thyroxine was given to normal human subjects, 30% of the radioactivity accumulated in the urine and thyroid by the end of 24 hours, mostly in the form of nonhormonal iodine. At this same time, there was a significant concentration of radioactivity in the liver as determined by external counting.

Johnson and Albert (1951) also studied the distribution of tagged thyroxine in the rat. They confirmed the high concentration in liver and noted a high concentration of labeled thyroxine, diiodotyrosine and iodide^{131} in the kidney as compared with plasma. No appreciable radioactivity was found in striated muscle, and insignificant amounts were noted in lung, testis, and pancreas 5, 15, and 24 hours after administration. More recently, it has been shown that, in addition to the concentration of thyroxine in liver, there is a large amount secreted into the bile, most of which is reabsorbed from the small intestine (Albert, 1952, Albert and Keating, 1952, Briggs, Taurog and Chaikoff, 1953). From 100 to 130% of the administered radioactivity is secreted each hour, though only 3% is lost in feces during the same period (Keating and Albert, 1953).

Observations on the tissue distribution of triiodothyronine have been more limited. Keating and Albert (1953) compared the distribution of triiodothyronine with that of thyroxine, finding a similar enterohepatic circulation, but over a two-week period, thyroxine was concentrated more and retained more selectively by the liver. There was a higher rate of urinary excretion, gastric mucosal secretion, and thyroidal uptake of nonhormonal iodine when triiodothyronine was given, suggesting that it was more rapidly deiodinated.

It seemed to us that it was also important to make comparative studies, at shorter intervals, of the distribution of thyroxin and triiodothyronine and to give additional attention to those tissues with a small concentration of radioactivity, e.g., muscle. Studies of this nature were conducted in rats and are hereby reported.

METHODS AND MATERIALS

Sprague-Dawley rats, weighing from 150 to 250 gm., were used throughout the experiments. They were kept in a room maintained constantly at 80° F., and were fed a standard diet of Purina fox chow. The food intake was limited to 10% dextrose in normal saline during the 14 hours before each experiment.

Five to eight microcuries of L-thyroxine and l-3,5,3′ triiodothyronine, labeled with I^{131} (specific activity 17 to 30 millicuries per mg.) were injected into the tail vein, using ether anesthesia. For each substance, groups containing from 6 to 10 animals were sacrificed at 5, 15, 30 and 60 minutes, 3, 6 and 12 hours, and 3 and 6 days following injection. All animals were killed by exsanguination, the blood being placed immediately into 10% trichloracetic acid, except in the few instances where separation into plasma and cells was desired. One-half to one gm. portions of liver, muscle, kidney, spleen, and in some cases thyroid, intestine, ovary, heart and testis, were homogenized immediately in

10% trichloracetic acid. The homogenates were stirred, diluted to constant volume, and counted by means of a gamma well counter (Texas type, manufactured by Welch-Allyn). The mixtures were then centrifuged, and the precipitate washed twice with 5% trichloracetic acid. The washed precipitates were extracted three times with n-butanol and the pooled extracts again counted in the well counter.

All other tissues were dissolved in a constant volume of 30% KOH and counted directly, no butanol extractions being made. As shown in Figure 1 and in Tables 1 and 2, all tissues were analyzed 30 minutes after injection of the hormone; certain ones also were selected to be analyzed 60 and 180 minutes after injection.

The butanol extracts were considered to represent hormonal iodine. The difference between this fraction and the total was assumed to be iodide and possibly small amounts of monoiodo- and diiodotyrosine (Rall, 1950). By use of an identical dose as standard for each group, results were calculated in the following ways:

1. Per cent of total radioactivity injected, in each tissue
2. Counts per minute, per gm. of tissue
3. $$\frac{\text{"[T]"}}{\text{[B]}}\left(\frac{\text{Concentration in tissue}}{\text{Initial concentration in total body}}\right)$$

$$=\frac{\text{Per cent of total radioactivity}}{\text{Weight (gm.) of tissue}}\times\frac{\text{Body weight}}{100}.$$

RESULTS

A. *Comparison of the Distribution of Thyroxine and Triiodothyronine*

Thirty minutes following the administration of these hormones, the concentrations of radioactivity in various tissues of the rat were determined. As can be seen from Figures 1 and 2, the plasma level of triiodothyronine

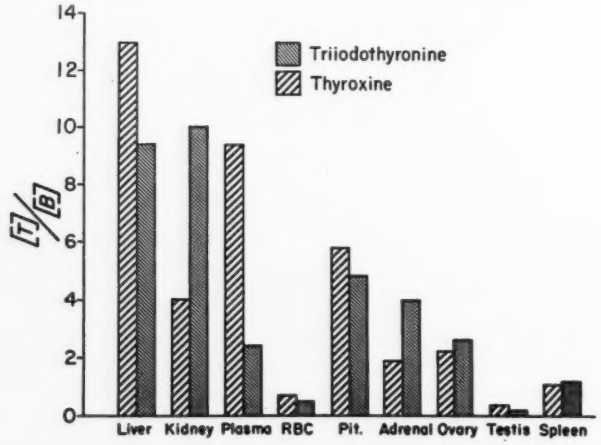

FIG. 1. Concentration of radioactivity thirty minutes following thyroxine and triiodothyronine administration. Statistically significant differences between the two hormones were present in all tissues except pituitary, ovary and spleen.

TABLE 1. CONCENTRATION OF RADIOACTIVITY ([T]/[B]) IN VARIOUS TISSUES REMOVED ONE HOUR FOLLOWING INJECTION OF LABELED HORMONE. THIS REPRESENTS TOTAL RADIOACTIVITY, AND IN SOME TISSUES, NOTABLY THYROID AND STOMACH, IS COMPOSED LARGELY OF NON-HORMONAL IODIDE

Tissue	[T]/[B] Triiodothyronine	[T]/[B] Thyroxine
Liver	5.80	9.7
Kidney	7.0	3.5
Muscle	.42	.29
Spleen	.97	1.11
Plasma	1.31	9.19
RBC	—	0.57
Lung	—	1.76
Large Intestine*	.44	.337
Small Intestine*	4.59	2.84
Stomach	1.26	2.52
Thymus	—	.38
Fat	.31	.29
Testes	—	.44
Ovaries	3.56	1.89
Brain	.30	.22
Thyroid	60.41	52.7
Pituitary	—	4.14
Adrenal	—	1.74

* Includes contents.

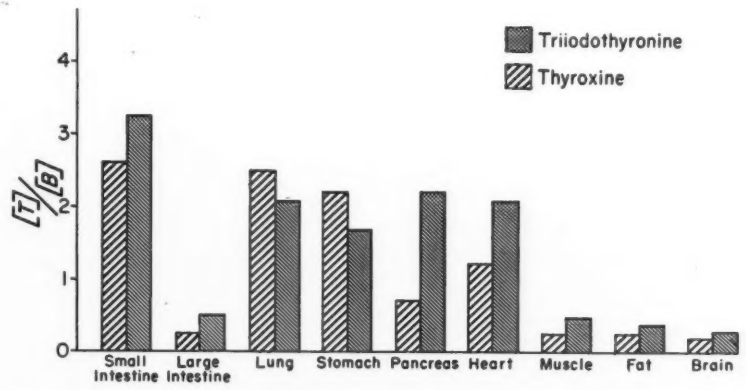

FIG. 2. Same conditions as Figure 1. Statistically significant differences were present in all tissues except fat.

was quite low in relation to thyroxine. Investigation of other tissues revealed that the concentration of radioactivity in the triiodothyronine group was higher in the adrenal gland, pancreas, heart, brain, kidney, large and small intestines, and muscle, while the concentration in the thyroxine group was higher in lung, stomach, testis, and liver. There was no significant difference between concentrations in the pituitary, ovary, spleen, or fat.

TABLE 2. SIMILAR DATA TO TABLE 1, BUT OBTAINED FROM TISSUES REMOVED THREE HOURS FOLLOWING INJECTION OF HORMONE

Tissue	[T]/[B] Triiodothyronine	[T]/[B] Thyroxine
Liver	3.3	7.8
Kidney	4.3	3.0
Muscle	.48	.37
Spleen	.70	.75
Plasma	.97	6.09
RBC	—	.79
Lung	—	1.59
Large Intestine*	1.0	1.45
Small Intestine*	5.2	5.13
Stomach	6.6	2.66
Thymus	—	.38
Fat	—	.34
Testes	.254	.71
Ovaries	1.16	1.88
Brain	—	.27
Thyroid	19.68	13.5
Pituitary	—	3.03
Adrenal	1.89	1.25
Pancreas	—	0.58

* Includes contents.

Concentrations were then determined at various other times after injection. Tables 1 and 2 show the distribution at 60 and 180 minutes, represented as tissue concentration "[T]/[B]." Figure 3 demonstrates the distribution of radioactivity in some tissues at 30, 60 and 180 minutes, recorded as percent of initial dose in the entire organ. As would be expected, a gradual drop, paralleling that of plasma, occurred in most of the tissues studied, the main exceptions being the thyroid, GI tract (with contents) and some of the tissues with low hormonal concentrations such as testis, muscle, brain and fat. The amount of radioactivity in the plasma following thyroxine administration remained much higher than after triiodothyronine, at 3 hours being 18% of the dose compared with 2.9%.

B. *Concentration in Liver, Kidney, Muscle and Blood, as a Function of Time*

Certain tissues were selected for more detailed study of their ability to concentrate the two hormones. Figures 4, 5, 6 and 7 show the comparative concentrations in blood, kidney, liver and muscle respectively, from 5 minutes to 12 hours after injection. It can be seen that at 5 minutes, triiodothyronine was at a concentration significantly higher than thyroxine in

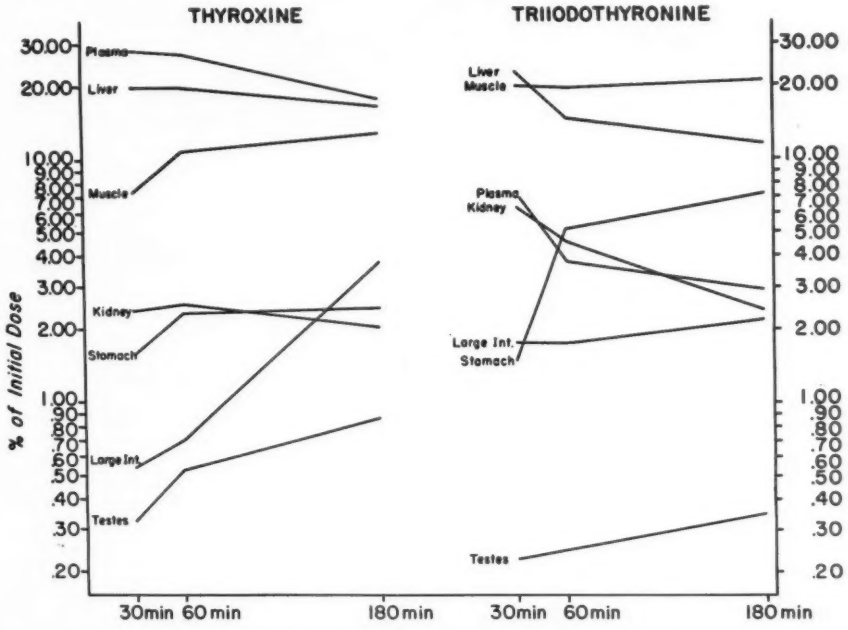

FIG. 3. Per cent of initial dose present in total mass of selected organs. Muscle mass was estimated on the basis of 90 gm. muscle per 200 gm. body weight.

liver, kidney and muscle ($0 < 0.001$). In liver, this difference reversed itself by 15 minutes, and from then on, thyroxine concentration was significantly greater ($p < 0.001$). In the kidney, the triiodothyronine level remained high for a longer period, being concentrated to 6 times its plasma level at 30 minutes, and 2.5 times the concentration of thyroxine in the kidney at the same time. This difference was statistically significant until the 12-hour point when the triiodothyronine level dropped below that of thyroxine, though not significantly so. In skeletal muscle triiodothyronine was present at significantly higher levels than thyroxine ($p < 0.001$) during the first six hours, then fell to a level insignificantly greater than that of thyroxine

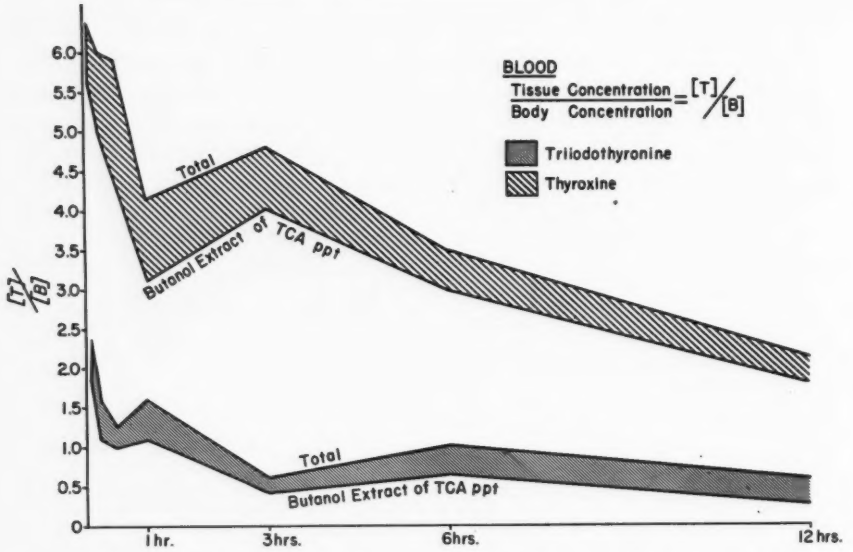

FIG. 4. Concentration of radioactivity in blood at various periods following administration of thyroxine and triiodothyronine. Shaded areas represent non-hormonal radioactivity, and the areas beneath indicate the concentration of labelled thyroxine or triiodothyronine respectively.

at 12 hours. Concentration of thyroxine in muscle also rose throughout the first six hours, then fell gradually.

Studies 3 and 6 days after injection of the two hormones revealed a constantly higher concentration of thyroxine in liver, kidney and blood (Fig. 8).

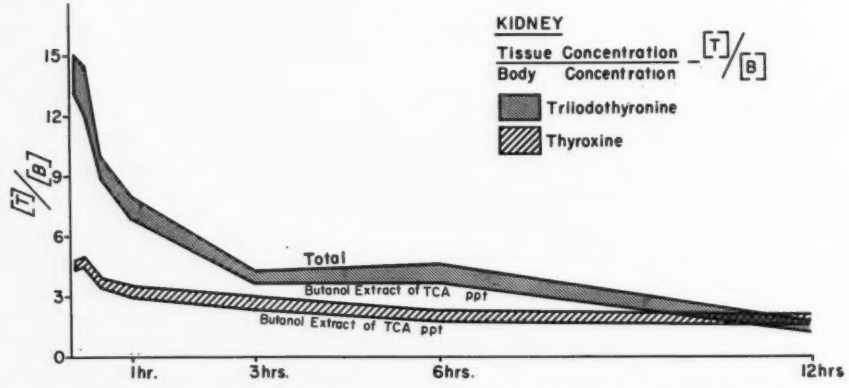

FIG. 5. Concentrations of labeled hormones in the kidney, charted as in Figure 4.

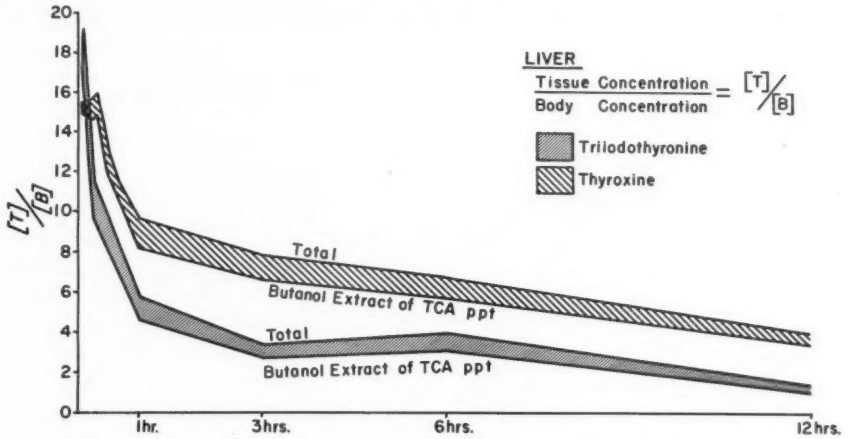

FIG. 6. Concentrations of labeled hormones in the liver, charted as in Figure 4.

DISCUSSION

It is recognized that the ability of thyroxine and triiodothyronine to concentrate in certain tissues does not necessarily mean that these tissues are more intimately involved in the metabolism and utilization of the hormones. The physiological effects may occur predominantly in muscle, where concentrations of hormone have proven to be quite low, and to a

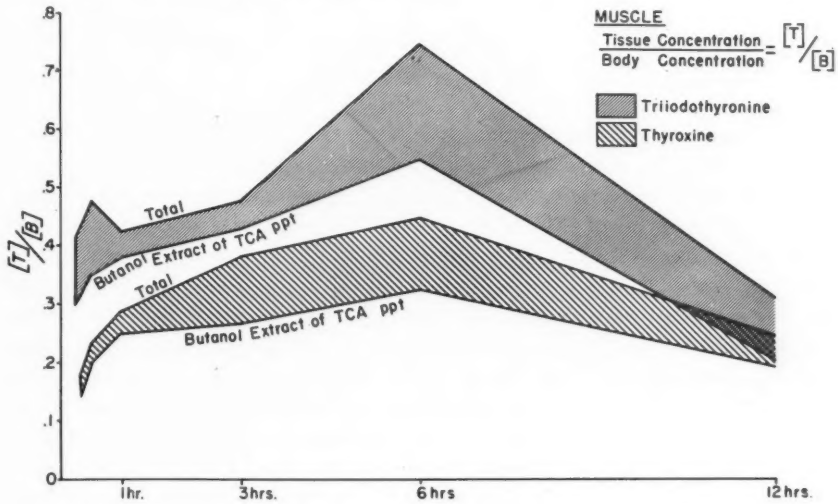

FIG. 7. Concentration of labeled hormones in skeletal muscle, charted as in Figure 4. Note that values of [T]/[B] are much lower than in the three previous figures.

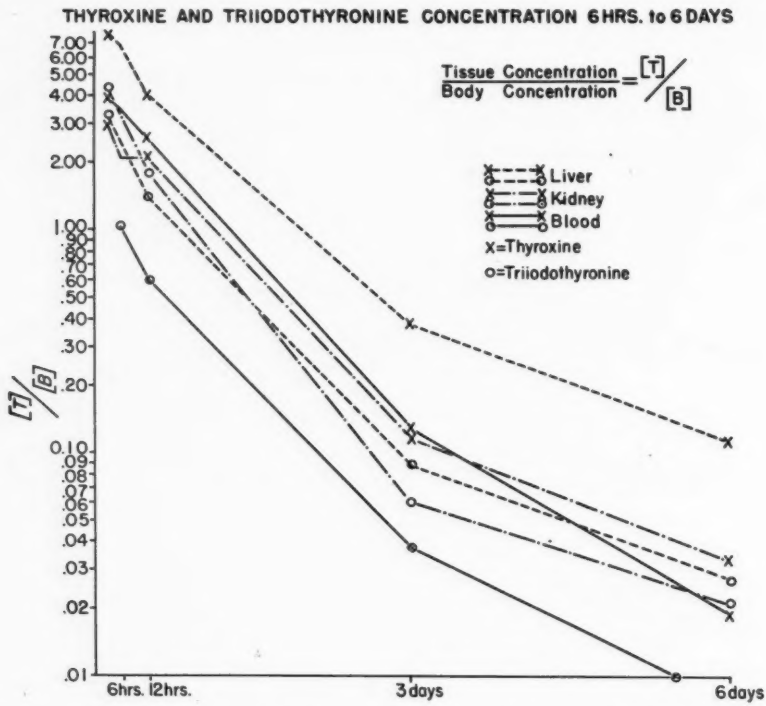

FIG. 8. Concentration of total radioactivity in liver, kidney and blood following thyroxine and triiodothyronine administration.

small extent in liver, where concentrations are much higher.

Deiss, Albright and Larson (1953), by means of paper electrophoresis, have recently shown that, *in vitro*, triiodothyronine was more loosely bound than thyroxine to certain plasma proteins (especially α globulin). In addition, thyroxine tended to displace triiodothyronine from these proteins. Gordon, Gross, O'Connor and Pitt-Rivers (1952) have also found thyroxine to be associated with α globulin, but have not been able to find triiodothyronine associated with this specific protein in the circulation (Gross and Pitt-Rivers, 1953 c). There seems to be a good chemical reason, therefore, for the higher blood levels of thyroxine in plasma. We do not mean to imply that this is the main reason triiodothyronine leaves the blood more rapidly than thyroxine. A more rapid uptake and degradation of triiodothyronine by tissues is probably the primary cause of lower blood levels.

Previous work has produced evidence in animals and man that thyroxine is concentrated and presumably stored in liver (Johnson and Albert, 1951,

Myant and Pochin, 1950, Rawson *et al.*, 1953). Our long term studies (3–6 days) show results that are consistent with these findings. It is evident from inspection of the short term data, however, that, although the difference in concentration of the two hormones in liver is significant statistically, this difference is slight in relation to the total amounts of each hormone. In the case of both substances a similar and rapid fall in concentration occurred during the early period. This fall was greater than the fall in plasma levels during the same period, and continued until the time (about three hours) when enterohepatic circulation is said to reach equilibrium for both substances (Keating and Albert, 1953). It was during, and for a short time after, the period of equilibration of the enterohepatic circulation, that the relative differences in triiodothyronine and thyroxine concentration became greater. As can be seen from Figure 6, the absolute difference in concentration of the two substances remained about constant during the first few hours, so that thyroxine became proportionately more concentrated than triiodothyronine. At about the 12-hour point, the biological decay entered a new and slower phrase, paralleling roughly the decay in plasma levels. During this slow phase, the ratio of thyroxine concentration to triiodothyronine concentration remained almost constant, as can be seen from the parallel decay rates on the semilogarithmic scale (Figure 8).

The appreciably higher concentration of triiodothyronine in the kidney during the early period is of great interest. The possibility of concentration for purpose of excretion was considered, but it has been shown previously that thyroxine, diiodotyrosine, and mono-iodotyrosine are not excreted in significant amounts (Myant and Pochin, 1950, Rall, 1950, Albert, 1951, Hogness, Wong and Williams, 1954) and our data show that relatively little triiodothyronine is excreted (Table 3). It can only be speculated whether the kidney concentrates triiodothyronine for the purpose of storage, or for utilization and degradation. We found this hormone to be present in the renal cortex in a concentration about 30% greater than in the medulla, but a similar difference is found with thyroxine (unpublished data).

The calorigenic action of both hormones should involve muscle to a great degree. Although absolute concentrations of hormone in muscle were small,

TABLE 3. URINARY EXCRETION OF RADIOACTIVITY FOLLOWING TRIIODOTHYRONINE ADMINISTRATION[3]

Time	% of initial dose I^{131} in urine		% of I^{131} in urine as hormone
	Total	Hormonal	
0–24 Hrs.	25.7%	2.2%	8.42%
24–48 Hrs.	7.4%	0.6%	8.11%

[3] Determined by methods identical to those used by Hogness, Wong and Williams (1954) in measuring urinary excretion of radioactivity following radio-thyroxine administration.

the total muscle mass contained 10% of radioactivity after thyroxine and 20% after triiodothyronine during the first few hours following injection (Fig. 3). The increased concentration of triiodothyronine over thyroxine seems to be consistent with its more rapid metabolic effect. This difference seems even more significant if it is kept in mind that the concentration of thyroxine in the perfusing blood is much greater than that of triiodothyronine. It is surprising that, although higher, the triiotlothyronine level in muscle is parallel to that of thyroxine during the period studied. There was evidently a more rapid rise of triiodothyronine during the first few minutes after administration, followed by a slower rise with a slope almost identical to that of thyroxine, suggesting that, after the first few minutes, a similar turnover rate of hormone occurs. The relatively large non-precipitable fraction observed in muscle after administration of both hormones is considerably less than the corresponding fraction in blood, and probably represents passive diffusion into muscle tissues rather than indicating a large degree of local deiodination of hormone.

In most of the remaining tissues, differences in thyroxine-triiodothyronine concentrations were significant statistically. Higher counts in the stomach and thyroid after administration of triiodothyronine could be attributed to the large amounts of free iodide concentrated in these organs. The higher level of radioactivity after injection of triiodothyronine, in small and large intestine, we presume again to be due to the increased amount of iodide released, since thyroxine and triiodothyronine apparently are released to the bile by the liver in roughly equal amounts, and fecal excretion of radioactivity is similar (Keating and Albert, 1953).

As would be expected, levels in most tissues dropped more or less parallel to that of plasma. The exceptions, other than the special tissues discussed, were among the tissues with very low concentrations, such as testis, fat, and brain, where concentrations rose gradually over the three hour period. As in the case of muscle, these rises may indicate a rather poor and slow tendency for hormone concentrations to equilibrate with those of blood. However, equilibration with blood levels is not the only factor, since in brain and fat, as in muscle, triiodothyronine concentration was higher. This paradox might be explained by speculating that triiodothyronine crosses the cell membrane more easily than thyroxine.

SUMMARY

Comparisons were made, at various time intervals, of the distribution in rat tissues of L-thyroxine and l-3,5,3′ triiodothyronine, labelled with equal amounts of I^{131} with similar specific activity.

At each interval studied, the concentration of triiodothyronine in plasma was much lower than that of thyroxine and somewhat lower in liver, except

for an unexplained, but significantly higher, concentration 5 minutes following administration.

Triiodothyronine was present in higher concentrations in kidney during the first twelve hours after administration; its concentration then dropped to below that of thyroxine.

In skeletal muscle, concentrations of both hormones were relatively low, but the level of triiodothyronine was significantly higher than that of thyroxine during the first twelve hours. These concentrations rose gradually to a peak at six hours and then fell slowly. Relatively large amounts of iodide were observed to be present.

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EFFECT OF THYROXINE ON THE WEIGHT AND COMPOSITION OF MUSCLE, PELT AND OTHER TISSUES IN YOUNG HYPOPHYSECTOMIZED RATS

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RECENTLY it was observed that the increase in weight of striated muscle in response to growth hormone administration was greater in hypophysectomized rats than in thyroidectomized rats (Scow, 1953). The difference in response between the two groups of animals was assumed to be due to the secretion of thyroid hormone, although diminished, in the hypophysectomized rat.

The purpose of the present study was to determine if thyroxine given alone could effect a muscle growth response in hypophysectomized rats. Earlier, Ray *et al.* (1950) had reported a small increase in body weight and skeletal length of thyroxine-treated hypophysectomized rats. In the present experiments, not only were the findings of Ray *et al.* confirmed, but it was observed that the weight responses of skeletal and cardiac muscle, thymus, and pelt were proportionally greater than that of all other tissues.

EXPERIMENTAL

Male Holtzman rats at 21–23 days of age were hypophysectomized under sodium pentobarbital-ether anesthesia. This report includes only those operated animals in which total extirpation of the pituitary gland was achieved as ascertained by the absence of pituitary tissue in the region of the sella turcia (visualized with 9× magnification) and by atrophy of the target organs (thyroid, adrenals, testes, and accessory sexual tissue). The daily dosage of thyroxine, given subcutaneously, was 2.5 μg. per rat. A fresh solution of 2.5 mg. of DL-thyroxine (Hoffman-LaRoche) in 100 ml. of 0.001 N NaOH was made up every fourth or fifth day.

The rats in Experiment A were injected for 56 days, beginning on the fourth postoperative day, and were fed an adequate purified diet (4279) *ad libitum* which was supplemented twice weekly with 2 mg. vitamin E given by pipette (Scow, 1952). In Experiment B, the rats were injected for 46 days, beginning on the seventh postoperative day, and were fed, *ad libitum*, a purified diet (4370) similar to the above diet except that

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[1] The technical assistance of Marian Hellstrom and Susie Hagan is gratefully acknowledged.

vitamin E was added, 50 mg./kg. of diet. In Experiment B, food intake by each rat was measured during the first 21 days of the injection period.

On the day following the last injection, each rat was exsanguinated through a large cardiac incision while under chloroform anesthesia and subjected to immediate dissection and weighing of tissues. The water, protein, and fat content of the tissues was determined by a modification of the method of Kochakian and Stettner (1948; Scow, 1951) and collagen by the method of Lowry *et al.* (1941). The various nitrogen fractions in the thigh muscle were determined according to a modification of a method described earlier (Scow, 1953), which was based on the procedure of Weber and Meyer (1933). The muscle was cut into small pieces with scissors and then ground with sea sand in a chilled porcelain mortar and pestle. The sample was then treated with Weber's solution (Weber and Meyer, 1933). Myosin[2] was precipitated from the Weber's extract by changing the pH from 9.2 to 6.8 and by diluting the KCl concentration from 0.6 M to 0.03 M. The fraction remaining in solution was designated as "water soluble" nitrogen. The stroma fraction was not soluble in Weber's solution. The thigh muscle from the other side of each animal was used for the determination of water and total protein content.

The various nitrogen fractions in the pelt were isolated according to the technique of Jacobs (1949). The pelt was cut into small pieces and treated with 0.1% HCl for 7 hours at 85° C. Elastin and keratin, including hair, were not solubilized by this procedure and were removed by filtration. "Globular" proteins were precipitated from the filtrate by adding trichloroacetic acid to a concentration of 3% and total proteins by adding trichloroacetic acid to a concentration of 30%. The difference between the latter two fractions represented collagen. The non-protein fraction was not precipitated by trichloroacetic acid.

RESULTS

The effect of thyroxine administration, 2.5 μg. daily, on body weight and body length in hypophysectomized rats is recorded in Table 1. The body weight gain during the injection period was doubled in both experiments as the result of thyroxine. The nose-tail length was also significantly greater in the treated than in the untreated hypophysectomized rats. During the second week of injections, the injected animals were easily distinguishable from the controls by their increased muscle tone, increased activity, and luxuriant hair growth.

The cumulative body weight gain and daily food consumption in Experiment B is recorded in Figure 1. The dip in the body weight curve of the thyroxine-treated animals did not occur in Experiment A and is unexplained. There was a time lag of at least 3 days before the daily food consumption and daily body weight gain were significantly increased following the onset of the thyroxine injections. From then on, the daily food consumption of the treated rats increased and that of the hypophysectomized controls decreased. During the first 10 days following the onset of thyroxine treatment, the body weight gain per gram of food eaten was 0.27 gm. in the

[2] Although this fraction is probably a mixture of myosin and actomyosin, for the sake of brevity it will be called myosin.

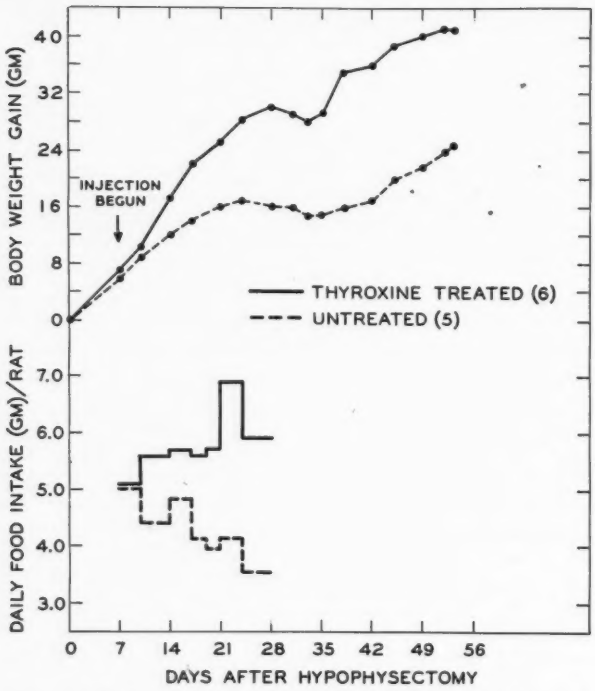

FIG. 1. Experiment B. Effect of thyroxine on the body weight gain and daily food consumption of hypophysectomized rats.

TABLE 1. EFFECT OF THYROXINE ON BODY WEIGHT AND BODY LENGTH IN HYPOPHYSECTOMIZED RATS

	No. of rats	Body weight (gm.)[a]		Nose-tail length (cm.)[a b]	
		Final	Gain	Final	Gain
Experiment A					
Hypophysectomized					
Control	6	67.8± 4.0	21.0± 2.0	25.1±0.4	5.8±0.5
Treated[c]	7	82.3± 2.0	42.7± 2.4	28.2±0.5	9.5±0.8
Normal					
Control	7	291.0±15.0	242.0±13.0	42.0±0.8	24.0±0.7
Experiment B					
Hypophysectomized					
Control	5	68.0± 2.4	18.8± 1.8	24.6±0.4	—
Treated[d]	6	79.6± 4.3	35.8± 2.8	28.6±0.4	—

[a] Mean ± standard error (S.E.); $S.E. = \frac{\text{range}}{\text{number of animals}}$ (Mantel, 1951).

[b] Measured to nearest 0.5 cm. while the rat was anesthetized and lying in a supine position.

[c] Experiment A: Injected daily with 2.5 μg. DL-thyroxine subcutaneously for 56 days beginning on 4th postoperative day.

[d] Experiment B: Injected daily with 2.5 μg. DL-thyroxine subcutaneously for 46 days beginning on 7th postoperative day.

treated and 0.17 gm. in the hypophysectomized controls, and during the subsequent 11 days, 0.10 gm. and 0.07 gm., respectively.

The weights of various organs are given in Table 2. The gain in weight of lungs, liver, kidney, spleen, and gastrointestinal tract in the thyroxine-treated hypophysectomized rats was proportional to the gain in body weight. The increased weight of the head (including brain and jaw muscles) and of the hind leg bones in the treated rats was so small that the proportionality of these tissues to body weight was significantly decreased when compared to the hypophysectomized controls. With the exception of the thymus gland, which was doubled in weight, there was no effect of thyroxine on the weights of the endocrine glands and of the accessory sexual tissues. Although the data for the normal age controls are presented in Table 2 and subsequent tables, it is not proper to compare them directly with the data for the dwarfed rats because of the marked effect that body size has on some of these measurements, such as the organ/body weight ratios (Donaldson, 1924). An example of this is the difference in the thigh muscle /body weight ratio between the 21 day and 81 day old normal male rats, 2.45% vs. 4.34%, recorded in Table 3.

TABLE 2. EFFECT OF THYROXINE ON THE WEIGHT OF VARIOUS ORGANS AND ENDOCRINE GLANDS IN HYPOPHYSECTOMIZED RATS
Experiment A[a]

	Hypophysectomized		Normal control
	Control	Treated	
No. of rats	6	7	7
Body weights[b]	65.2 gm.	79.7 gm.	284.0 gm.
Structure	% of body weight	% of body weight	% of body weight
Lungs	1.02 ±0.08[c]	0.96 ±0.06[c]	0.68 ±0.06[c]
Liver	4.17 ±0.21	4.18 ±0.12	3.62 ±0.08
Kidney (1)	0.34 ±0.02	0.38 ±0.02	0.38 ±0.02
Spleen	0.34 ±0.04	0.35 ±0.05	0.25 ±0.02
Gastro-intestinal tract (empty)	5.57 ±0.27	5.17 ±0.17	2.95 ±0.75
Head[d]	11.3 ±0.5	9.6 ±0.2	5.7 ±0.2
Bone[e]	0.97 ±0.02	0.89 ±0.02	0.74 ±0.02
Hair and skin[f]	19.5 ±0.7	21.0 ±0.4	19.8 ±0.5
	mg.	mg.	mg.
Thymus	116.0 ±25.0	236. ±22.	456. ±62.
Thyroid	3.7 ± 0.3	3.8 ± 0.1	25.3 ± 2.4
Adrenal (1)	4.6 ± 0.4	3.9 ± 0.6	19.4 ± 1.5
Testis (1)	50.4 ± 8.7	46.0 ±10.0	1,540. ±43
Seminal vesicles	4.6 ± 0.2	4.3 ± 0.5	330. ±33
Ventral prostate	3.7 ± 0.5	3.3 ± 0.5	380. ±24

[a] See footnote c of table 1.
[b] Body weight less weight of gastrointestinal tract contents.
[c] Mean ± standard error.
[d] Included skull, brain and jaw muscles.
[e] Unilateral; included femur, tibia, fibula and knee joint.
[f] Included all hair and skin except that on nose tip, paws, and tail.

TABLE 3. EFFECT OF THYROXINE ON WEIGHT OF VARIOUS MUSCLES IN HYPOPHYSECTOMIZED RATS

Group	No. of rats	Body weight[a] gm.[d]	Thigh muscle[b] (unilateral)		Heart		Levator ani muscle[c]
			gm.[d]	% of body weight[e]	mg.[d]	% of body weight[e]	mg.[e]
Experiment A[f]							
Hypophysectomized							
Control	6	65	2.06	3.14±0.09	248	0.384±0.016	9.2±1.5
Treated	7	80	2.89	3.65±0.05	374	0.472±0.015	8.7±1.6
Normal							
Control							
21 days old	5	41	1.00	2.45±0.04	—	—	—
81 days old	7	284	12.52	4.34±0.09	982	0.338±0.006	116.0±7.7
Experiment B[f]							
Hypophysectomized							
Control	5	68	2.20	3.23±0.09	277	0.407±0.013	—
Treated	6	80	2.79	3.50±0.08	412	0.515±0.023	—

[a] Body weight minus weight of gastrointestinal tract contents only in Experiment A.
[b] Thigh consisted of quadriceps, hamstring, adductor, and gluteal muscles, and related tendons, fascia, nerves, fat, and blood vessels.
[c] As described by Eisenberg and Gordan (1950).
[d] Mean
[e] Mean ± standard error.
[f] See footnotes c and d of Table 1.

Thyroxine administration resulted in a marked increase in the weight of the heart and thigh muscles in hypophysectomized rats, which was proportionally greater than the increase in body weight (Table 3). When one compares the hypophysectomized controls with the 21 day old normals, which are preoperative controls, it is evident that the thigh muscle has doubled in weight since the hypophysectomy. Thyroxine administration had no effect on the weight of the levator ani muscle.

The increased weight of the thigh muscle in the thyroxine-treated animals was associated with a significant gain in the total amount of protein and in the amount of muscle protein per unit body weight (Table 4). Thyroxine administration had a slight effect on the water/protein ratio in thigh muscle in only one of the two experiments. Since there was no significant gain in collagen and there was a gain in the total amount of protein in the thigh muscle, the collagen content in per cent of total muscle protein was reduced in the treated animals (Experiment A) ($P<0.01$). There was no significant difference between the two groups of hypophysectomized rats in the proportion of the three major groups of proteins in the thigh muscle (myosin, "water soluble" nitrogen (included sarcoplasmic protein and nonprotein nitrogen), and stroma) (Experiment B).

Thyroxine treatment had no effect in hypophysectomized rats on the amount of protein in the hind leg bones (Table 5). Although the total amount of protein, water, and fat in the carcass was greater in the treated

TABLE 4. EFFECT OF THYROXINE ON CHEMICAL COMPOSITION OF THIGH MUSCLE[a] IN HYPOPHYSECTOMIZED RATS

Group	No. of rats	Water[b]	Protein[b,c]			Water/protein ratio[b]	Collagen[b]	
		% of wet weight	% of wet weight	Total mg.	Mg./100 gm. body weight[d]		Total mg.	% of total protein
Experiment A[e]								
Hypophysectomized								
Control	6	73.7±0.4	19.2±0.2	400± 33	601±25	3.84±0.06	22.9± 2.3	5.92±0.26
Treated	7	73.3±0.3	19.4±0.2	570± 20	720±12	3.78±0.03	27.0± 1.6	4.89±0.15
Normal								
Control								
21 days old	6	76.4±0.9	17.5±0.2	170± 7	540±12	4.39±0.12	—	—
81 days old	6	73.1±0.3	21.2±0.3	2,690±150	942±30	3.45±0.04	181.0±12.0	6.57±0.34
							% of total nitrogen[b]	
							Myosin N	"Water soluble" N	Stroma N
Experiment B[e]									
Hypophysectomized									
Control	5	74.0±0.4	19.2±0.1	427±23	627±19	3.82±0.06	23.2±1.1	49.1±1.3	20.8±0.3
Treated	6	72.9±0.7	20.8±0.2	566±27	741±23	3.51±0.04	23.6±0.9	47.3±0.4	21.5±0.9

[a] Unilateral; consisted of quadriceps, hamstring, adductor, and gluteal muscles, and related tendons, fascia, nerves, fat, and blood vessels.
[b] Mean ± standard error.
[c] Protein = 6.25 × nitrogen.
[d] Body weight less weight of gastrointestinal tract contents only in Experiment A.
[e] See footnotes c and d of Table 1.

animals, the percentages of each and the water/protein ratio were unchanged. The protein content of the pelt of the thyroxine-treated hypophysectomized rats was markedly increased both in total amount and in the amount per unit body weight. The protein gain in the hair and skin ac-

TABLE 5. EFFECT OF THYROXINE ON CHEMICAL COMPOSITION OF BONE, CARCASS, AND PELT IN HYPOPHYSECTOMIZED RATS

Group	No. of rats	Water[a]	Protein[a,b]			Water/protein ratio[a]	Fat[a]
		% of wet weight	% of wet weight	Total gm.	gm./100 gm. body weight[c]		% of wet weight
Experiment A[d]			Bone[e]				
Hypophysectomized							
Control	6	49.6±0.9	19.7±0.2	0.13±0.01	0.190±0.004	2.49±0.06	—
Treated	7	47.8±1.2	20.2±0.3	0.15±0.01	0.180±0.006	2.37±0.08	—
Normal							
Control							
81 days old	7	46.6±1.3	19.7±0.4	0.43±0.02	0.150±0.006	2.35±0.08	—
			Carcass (eviscerated)[f]				
Hypophysectomized							
Control	6	60.1±1.1	17.9±0.2	8.80±0.53	13.65±0.16	3.37±0.06	12.8±0.7
Treated	7	60.0±0.9	19.1±0.5	11.68±0.24	14.69±0.24	3.13±0.05	12.3±0.8
Normal							
Control							
81 days old	6	61.2±0.9	20.5±0.3	47.69±3.27	16.58±0.22	3.14±0.06	10.8±1.1
			Pelt[g]				
Hypophysectomized							
Control	6	39.5±1.8	20.5±0.3	2.21±0.16	3.39±0.05	1.93±0.10	—
Treated	7	41.6±1.9	22.9±0.9	3.42±0.08	4.31±0.14	1.82±0.08	—

[a] Mean ± standard error.
[b] Protein = 6.25 × nitrogen.
[c] Body weight less weight of gastrointestinal contents.
[d] Injected daily with 2.5 μg. thyroxine for 56 days from 4th postoperative day.
[e] Unilateral; included femur, tibia, fibula, and knee joint.
[f] Included skin, hair, head, and dissected limbs.
[g] Included all skin and hair except that on nose tip, paws, and tail.

TABLE 6. EFFECT OF THYROXINE ON VARIOUS NITROGEN FRACTIONS OF THE PELT[a] IN HYPOPHYSECTOMIZED RATS

Group	No. of rats	Total protein[b,c]		% of total nitrogen[c]			
		gm.	gm./100 gm. body weight	Keratin-elastin N[d]	Globular protein N	Collagen N	Nonprotein N
Experiment B[e]							
Hypophysectomized							
Control	5	2.94±0.21	4.34±0.17	48.9±0.8	14.0±1.3	21.2±0.5	15.9±1.1
Treated	6	4.27±0.19	5.40±0.19	50.6±1.0	12.8±0.7	20.1±0.9	16.5±1.1

[a] Pelt included all hair and skin except that on nose tip, paws, and tail.
[b] Protein = 6.25 × nitrogen.
[c] Mean ± standard error.
[d] The chief component of this fraction in the rat pelt is hair.
[e] Injected daily with 2.5 μg. DL-thyroxine for 46 days beginning on the seventh postoperative day.

counted for about one-third of the total protein gain in the carcass of the treated animals. The data presented in Table 6 indicate that the various nitrogen fractions (hair, collagen, etc.) of the pelt were increased proportionally to each other.

DISCUSSION

These experiments clearly demonstrate that thyroxine can exert a pronounced protein anabolic effect in rats deprived of the pituitary hormones, including growth hormone. The growth of nearly all tissues was stimulated. Some tissues, such as heart and thigh muscle, were more responsive to the thyroxine injections, and others, such as bone, less responsive relative to the overall body weight gain when compared with growth of these tissues in normal rats of similar size. The only unresponsive tissues were the target organs which are dependent, directly or indirectly, on specific trophic hormones of the pituitary gland. The increment in carcass fat content was proportional to the body weight gain in the treated animals.

Selection of the proper dosage of thyroxine is apparently very important if one desires to demonstrate the protein anabolic effect of thyroxine in the absence of pituitary secretions. The daily dosage of thyroxine injected in this study and in that of Ray *et al.* (1950), in both of which growth in hypophysectomized rats was stimulated, was 2.5 to 3.0 μg. In several other studies in which larger amounts of thryoxine were administered, 5.0 μg. or more per day, growth was not stimulated (Becks *et al.*, 1946; Rupp *et al.*, 1949). Reineke *et al.* (1945) reported that the daily thyroid secretion in intact rats, as determined by the goiter prevention test, was equivalent to 5.0 μg. DL-thyroxine per 100 gm. body weight. In the present experiments, the average daily dosage per 100 gm. body weight was 4 μg. DL-thyroxine.

Based on the marked increase in organ/body weight ratios for heart and thigh muscle, thyroxine can be considered to have myotrophic activity. The weight of the levator ani muscle, however, was not affected by thyroxine. Earlier, it was observed that testosterone in the absence of the pi-

tuitary gland stimulated growth of the levator ani and perineal muscles but had no effect on the heart and thigh muscles (Scow, 1952). In a recent study in rats deprived of both the thyroid and pituitary glands (Scow, in press), growth hormone stimulated the growth of all of these muscles. These observations suggest that the myotrophic activity of some hormones is specific for certain muscles. Grouping muscles according to histological characteristics is not sufficient for this type of classification, as evidenced by the marked differences in growth response to various hormones between the leg and sexual muscles, all of which are striated muscles. It may be that grouping muscles according to function would be more helpful in describing the myotrophic activity of hormones.

The protein increment in the pelt accounted for about one-third of the increased protein content of the carcass of the thyroxine-treated rats. In the earlier part of the injection period, luxuriant growth of hair was noted. The chemical analyses indicated that all fractions of the pelt were proportionally increased. The increment in pelt protein content was about 60% more than that which would be expected on the basis of increased surface area in the injected animals. These experiments demonstrate that the stimulatory effect of thyroxine on the pelt, including hair, can occur in the absence of the pituitary gland. It is of interest to compare the effect of thyroxine in hypophysectomized rats with the effect of growth hormone in thyroidectomized rats on skin collagen (Scow, 1951). In the former the increment in skin collagen was proportional to that in the total skin proteins whereas in the latter, the increase in skin collagen was proportionally greater than the increase in the total skin proteins.

Salter and Best (1953) recently reported that insulin stimulated growth in hypophysectomized rats. It is interesting to compare their study with the observations on thyroxine reported in the present paper. The dosage of insulin and thyroxine given to the hypophysectomized rats was probably the maximal amount which could be tolerated without gross catabolic effects. Although the difference in body weight gain between treated and untreated rats was greater in the insulin experiments, the increased protein retention due to therapy in both studies was the same, about 3 gm. per rat. A major part of the difference in body weight gain between the two treatments can be accounted for by the greater increase in carcass fat in the insulin-treated than in the thyroxine-treated hypophysectomized rats. Relative to body weight, the heart weight was larger in the thyroxine-treated and the liver weight larger in the insulin-treated rats. It should be noted that the body weight gain and protein retention in hypophysectomized rats treated with either insulin or thyroxine is very small compared to that which can be observed, on either a daily weight gain or maximal weight gain basis, in hypophysectomized rats treated with growth hormone alone.

For example, Simpson *et al.* (1949) observed an average body weight gain of 341 gm. in hypophysectomized rats treated with pure pituitary growth hormone.

In other studies, it has been observed that growth in hypophysectomized rats is practically zero when deprived of either insulin (Salter and Best, 1953) or thyroid hormone (Evans *et al.*, 1939; Scow, in press). Growth hormone in the absence of insulin is reported to be ineffective in producing nitrogen retention (Milman *et al.*, 1951). The growth response of completely thyroidectomized rats to growth hormone is also very small (Scow *et al.*, 1949). When either the insulin or thyroid deficiency is corrected, responsiveness of the hypophysectomized rat to growth hormone is greatly enhanced (Milman *et al.*, 1951; Simpson *et al.*, 1950). It would seem then that both insulin and thyroid hormone are equally important for the normal and optimal growth response of somatic tissue to growth hormone administration.

SUMMARY

Administration of thyroxine, 2.5 μg. daily, doubled the body weight gain in male rats hypophysectomized at weaning. The carcass protein gain as the result of thyroxine therapy was 3 gm. per rat. The increment in body fat was proportional to that in body weight. Some tissues were considerably more responsive than others to thyroxine injections. The proportion of thigh muscle protein to body weight was increased 20% and that of the total pelt (skin and hair) protein increased 25%. The pelt protein increment accounted for 30% of the total protein gain in the treated animals. Determination of various nitrogen fractions in muscle and pelt indicated that all fractions were proportionally increased, with the exception of muscle collagen which was decreased. The heart/ body weight ratio in thyroxine-treated rats was also increased whereas the proportion of hind-leg bone and of head weight to body weight were decreased. With the exception of thymus, which was doubled in weight, there was no effect of thyroxine on the weight of the endocrine glands or the accessory sexual tissue. Thyroxine administration had no effect on the weight of the levator ani muscle in hypophysectomized rats.

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INFLUENCE OF ADRENAL CORTICAL HORMONES UPON THE LEVEL OF PLASMA AMINO ACIDS IN EVISCERATE RATS

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INTRODUCTION

AFTER removal of the liver, amino acids released from tissue proteins cannot be deaminated and therefore accumulate in the plasma. The rate of rise of the plasma amino acids may therefore be considered a rough indication of the net rate of protein breakdown. When adrenalectomized rats are eviscerated, the accumulation of plasma amino acids is depressed (Bondy, 1949; Ingle, Prestrud and Nezamis, 1948). In a previous communication it was claimed that the administration of large amounts of adrenal cortical extract to eviscerated rats with intact adrenals increased the rate of rise of plasma amino acids above control values within 4 hours (Bondy, 1949). Subsequent attempts to duplicate this experiment were unsuccessful. Further experiments were therefore carried out to determine whether adrenal cortical hormones can influence protein breakdown in the liverless animal, and to evaluate some of the factors concerned in the reaction.

METHODS

Two sets of experiments were carried out. In series one, attempts were made to duplicate the results of the previous experiments. Sprague-Dawley male rats, 300 gm. in weight or heavier, were functionally eviscerated according to the technique of Russell (1942). Glucose was given at the time of evisceration and at hourly intervals, in doses of 60 mg. subcutaneously, except where hydrocortisone in 5% glucose was given, since in this case the hydrocortisone injections also involved the administration of 50 mg. of glucose per hour. The animals were adjusted to a rectal temperature of 100–101° F. (37.8–38.4° C).

In series two, 250 gm. male rats of the Sprague-Dawley strain were totally eviscerated and adrenalectomized and were maintained by the intravenous injection of appropriate solutions, according to the technique described by Ingle and Nezamis (1948). The use of the thrombin-soaked gelatin sponge for hemostasis was omitted. The animals were kept in a temperature box at 26–27° C.

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At the end of the experiment the animals were bled from a large vessel (inferior vena cava in the first experiments, and abdominal aorta in the second), and the blood analyzed for amino acid nitrogen (Frame, Russell and Wilhelmi, 1943; Russell, 1944) and glucose (Nelson, 1944)—in series one, Hamilton and Van Slyke (1943) and Miller and Van Shyke (1936) in series two.

Control animals were run simultaneously with the experimental animals in each series. As a control for adrenal cortical extract, equal volumes of isotonic saline were given. In the case of six-fold concentrated adrenal extract, dissolved in 10% ethanol, the control consisted of 10% ethanol in saline. The control solution for hydrocortisone was 5% glucose with 1% ethanol.

The aqeuous adrenal cortical extract (Upjohn) has glycogen-depositing activity equivalent to 0.1 mg. hydrocortisone per ml. In some experiments the extract was concentrated to double strength. The six-fold concentrated extract was prepared by lyophilizing[2] a known volume of extract and taking the dry residue up in 1/60 the original volume of ethanol, after which the material could be brought to a final volume 1/6 of the original with saline.

Cortisone was prepared for intravenous use in 5% ethanol, and hydrocortisone was dissolved in 5% glucose and 1% ethanol.[3] Intramuscular cortisone was given as a microcrystalline suspension.

RESULTS

Adrenal cortical extract was without effect upon the plasma amino nitrogen concentration when given in two-or six-fold concentrated solution over a 4-hour period. The administration of glucose to animals receiving double-

TABLE 1. EFFECT OF ADRENAL CORTEX EXTRACT ON FUNCTIONALLY EVISCERATED RATS WITH INTACT ADRENALS. ALL ANIMALS WERE MAINTAINED AT A BODY TEMPERATURE OF 37° C.

Adrenal cortical extract			Glucose	Duration	Final concentration, mg. %							
					Glucose				Amino nitrogen			
Strength	Dose	Time			n	Control	n	ACE	n	Control	n	ACE
	ml.		mg./100 gm./hr.	hrs.								
×2	1.5	hourly ×3 subcut.	10	4	6	135 ± 12.7*	6	129 ± 8.0	6	17.95 ± .67	6	17.47 ± .94
			0	4					6	17.76 ± .63	6	17.50 ± .25
×6	1.5	hourly ×3 subcut.	20	4					4	29.4 ± 2.69	4	31.6 ± .50
Hydrocortisone alcohol 0.4 mg. one hour before evisceration, and +0.2 mg. hourly ×3 subcut.			17	4					6	25.6 ± 2.9	6	24.6 ± 3.6

* All values represent mean ± standard error.

strength extract failed to alter the amino acid concentration, nor did the adrenal extract alter the glucose tolerance of these animals (Table 1).

It seemed possible that the liver might be necessary for the action of the adrenal cortical hormones, and hydrocortisone was therefore given intra-

[2] We are indebted to Dr. Morris Tager for lyophilizing the extract.

[3] Generously donated by Dr. Elmer Alpert, Merck and Company.

TABLE 2. EFFECT OF ADRENAL CORTEX EXTRACT ON TOTALLY EVISCERATED RATS ADRENALECTOMIZED AT THE TIME OF EVISCERATION

Adrenal cortex extract			Glucose load		Duration	Final concentration, mg. %							
						Glucose				Amino nitrogen			
Conc.	Dose	Route		Insulin		n	Control	n	ACE	n	Control	n	ACE
	24 hr. ml.		mg./24 hr.	u/24 hr.	hrs.								
×1	20	Continuous I.V.	40	4	3	6	45 ± 5.9	6	50 ± 5.4	6	4.00± .11	6	4.23± .34
					6	6	50 ±12.9	6	71 ±12.5	6	4.30± .15	6	4.76± .31
×1	20	Injection	0	0	3	6	28 ± 2.4	6	32.3± 5.3	6	7.77± .34	6	7.45± .40
×1	2	I.V. Primer at Evisceration	0	0	3	7	34.4± 2.9	7	34.4± 2.0	7	7.24± .20	7	7.28± .23
	80	I.V. Continuous											
×2	1.5 hourly ×3	S.Q. Intermittent	0	0	3	6	36.8± 3.6	6	46.6± 4.3	6	8.77± .37	6	8.66± .43
					3*	13	32.6± 1.4	13	38.8± 2.2 (t=2.377)	13	11.62± .36	13	11.44± .37
×2	1.5 hourly ×6	I.V.	10	0	6*	6	64.0± 5.4	6	70.0± 5.2	6	24.87±1.0	6	24.16± .79

* These rats were maintained at a body temperature of 37° C. All others were maintained at an ambient temperature of 26–27° C.

muscularly one hour before evisceration and repeated at hourly intervals until sacrifice 4 hours after evisceration. Hydrocortisone failed to affect the plasma amino nitrogen levels in this experiment (Table 1).

A series of attempts to produce an effect of adrenal cortical extract within 3 or 6 hours after total evisceration and adrenalectomy are summarized in Table 2. Regardless of the dose of extract or the glucose load, no effect of the adrenal hormones could be demonstrated on either plasma amino acids or glucose tolerance.

Cortisone was given to totally eviscerated adrenalectomized rats in varying doses for various periods up to 48 hours (Table 3). In experiments lasting only 3 or 6 hours, no effect of cortisone was demonstrated; however, when the hormone was given for 24 hours there was consistent increase of the plasma amino nitrogen concentration. This response was not altered by increasing the glucose load. When insulin was given with glucose, the final concentration of amino nitrogen was significantly depressed both in control and hormone-treated animals, but cortisone continued to show an effect.

At 48 hours cortisone also increased the plasma amino nitrogen concentration, but the effect was less dramatic than at 24 hours.

Cortisone failed to alter the glucose tolerance when given for only 3 or 6 hours; however, when given for 24 hours without insulin it significantly depressed glucose tolerance at all glucose loads tested, except 16 mg./100 gm. per hour. When insulin was given with the glucose, an opposite effect was seen, and glucose tolerance was increased by cortisone. At 48 hours no significant effect of cortisone was demonstrated.

The failure of adrenal cortical hormones to affect either glucose or amino acid metabolism in animals studied 3 or 6 hours after adrenalectomy and

TABLE 3. EFFECT OF CORTISONE ON TOTALLY EVISCERATED RATS ADRENALECTOMIZED AT THE TIME OF EVISCERATION. TEMPERATURE 26–27° C.

Cortisone Dose/24 hr. and Interval	Glucose Load	Duration	Final concentration, mg. %							
			Glucose				Amino nitrogen			
			n	Control	n	Cortisone	n	Control	n	Cortisone
	mg./100 gm./hr.	hrs.								
6 mg. I.V. continuous	40+4 u insulin/24 hrs.	3	9	67± 5.9	9	59± 3.6	9	3.28± .09	9	3.42± .06
		6	11	66± 4.5	11	74± 5.3	11	3.53± .11	11	3.93± .09 (t=2.747)*
10 mg. intramuscular (5 mg. at evisceration+5 mg. 6 hrs. later)	8	24	10	77± 6.2	12	119± 8.2 (t=4.065)‡	13	25.09±1.20	15	29.93±1.02 (t=3.063)†
	12	24	7	106±19.3	7	212±16.5 (t=4.179)†	7	25.02±1.08	7	31.10±1.28 (t=3.626)†
	16	24	19	326±10.2	19	339± 9.2	19	23.75± .35	19	28.70± .55 (t=7.637)‡
	20	24	15	389±15.1	16	443±18.8 (t=2.3901)*	15	25.93± .62	16	30.48± .64 (t=5.1008)‡
	36+4 u	24	11	87± 6.8	11	62± 5.6 (t=2.836)*	11	6.28± .16	11	8.43± .21 (t=8.07)‡
	44+4 u	24	10	221±34.9	10	103±11.6 (t=3.210)†	10	5.34± .22	10	6.71± .17 (t=4.9068)‡
	60+4 u	24	11	570±54.1	11	274±33.3 (t=4.658)‡	11	6.78± .28	11	8.72± .22 (t=5.446)‡
10 mg. intramuscular (5 mg. at evisceration+5 mg. 6, 24, and 30 hrs. later)	36+4 u	48	13	82± 8.0	12	78± 4.4	13	14.83± .43	12	15.87± .58 (t=1.437)
	64+4 u	48	13	926±66.3	14	1029±84.9	13	15.53± .33	14	17.20± .62 (t=2.3664)*
	64+4 u	48	19	775±56.8	19	908±71.3	19	15.2 ± .44	19	16.5 ± .38 (t=2.2565)*

"t" values refer to significance of the difference between control and cortisone treated groups $\left(t = \frac{m_1 - m_2}{\sqrt{SE_1^2 + SE_2^2}}\right)$

* p .05.
† p .01.
‡ p .001.

evisceration raised the possibility that the trauma of evisceration prior to adrenalectomy might have caused so great a discharge of endogenous adrenal hormones that the maximum protein and carbohydrate effect was produced. The test substances could not, therefore, exert any effect until after the results of the endogenous hormone had worn off. In order to test this hypothesis, the experiments shown in Table 4 were done. The effects of adrenalectomy 24 hours before evisceration were compared with adrenalectomy at the time of evisceration. As a control a very small dose of hydrocortisone was given to some of the animals adrenalectomized 24 hours before evisceration.

It is clear from Table 4 that adrenalectomy performed at the time of evisceration does not alter either the plasma amino nitrogen or glucose tolerance, suggesting that three hours is too short a time for the effects of the operation to wear off. In contrast, adrenalectomy 24 hours before evisceration produces a significant depression of plasma amino nitrogen (p 0.01), confirming previous observations. This depression can be returned significantly toward normal by hydrocortisone; but with the small dose used, complete restoration to normal was not obtained. The effect on glucose tolerance was similar, except that the dose of hydrocortisone used in this experiment failed to alter the glucose tolerance of the adrenalectomized rats whether or not they had lost their adrenals before evisceration.

TABLE 4. COMPARISON OF THE EFFECTS OF ADRENALECTOMY 24 HOURS BEFORE EVISCERATION AND AT THE TIME OF EVISCERATION. THESE RATS WERE NOT FASTED BEFORE EVISCERATION

Operation	Adrenalectomized 24 hours before evisceration		Adrenalectomized at evisceration	Not adrenalectomized
	Hydrocortisone			
Steroid Treatment mg./100 gm./hr. i.v. ×3	0	.08	0	0
Glucose Load mg./100 gm./hr. i.v. ×3	20	20	20	20
Duration of Experiment hours (after evisceration)	3	3	3	3
Number of Rats	10	8	7	7
Plasma Amino Nitrogen mg./100 ml.	6.0 ± .43	7.5 ± .53	8.2 ± .85	11 ± 1.47
"p"		.05	.10	
Blood Glucose mg./100 ml.	96 ± 8.2	104 ± 10.0	147 ± 8.3	173 ± 18.3
"p"		.40	.30	

DISCUSSION

The present experiments demonstrate that under certain conditions adrenal steroids can alter protein and carbohydrate metabolism in the absence of the liver. The effect is easily demonstrated when the animals are deficient in the hormones, as a result of prior adrenalectomy. Even in animals which have been adrenalectomized at the time of evisceration, however, the effect can be shown if the study is continued long enough to permit the hormones secreted during the operation to be exhausted so that the effects of the test materials can become apparent.

On the other hand, no support was found for the hypothesis that the adrenal steroids (typified by hydrocortisone) must be metabolized by the liver in some way before they are physiologically active. The administration of the hormone an hour prior to evisceration did not increase its effectiveness.

It therefore appears that the carbohydrate and nitrogen effects of the adrenal steroids, like the effects of these substances on muscle work (Ingle, Nezamis and Meeks, 1953) can be produced in the absence of the liver.

Through the experiments the plasma amino nitrogen levels were independent of the amount of glucose given the animals. When insulin was given, however, the rise of amino nitrogen was reduced. These observations are consonant with those of Frame and Russell (1946), and Ingle, Prestrud, and Nezamis (1948) in the rat, and of Mirsky (1938), and Bollman, Flock, Grindlay, Mann, and Block (1953) in the dog.

The adrenal steroids had their expected effect of reducing glucose tolerance when given under certain conditions. Evisceration includes pancrea-

tectomy as well as hepatectomy. The reduction of glucose tolerance under these experimental conditions therefore occurs in insulin deficient animals. When insulin is given the effect is reversed, and the cortisone-treated animals become more tolerant of carbohydrate loads. Similar observations have been reported by Ingle and Nezamis (1948) who found that the glucose tolerance of adrenalectomized-eviscerated rats was less than that of eviscerated rats with intact adrenals when large glucose and insulin doses were used. The explanation for this apparent paradox is not clear, but it is possible that the improved circulation and general physical condition produced by the adrenal steroids in the adrenalectomized-eviscerated rat permit him to make a more adequate response to insulin than is possible in the adrenal-deficient animal. Ingle, Nezamis and Humphrey (1953) reported that cortisone and hydrocortisone have smaller effects than does adrenal cortical extract upon the glucose tolerance of the eviscerated rat. The larger doses of steroids used in the present study caused a significant response.

SUMMARY

Totally or functionally eviscerated rats were given adrenal cortical extract, cortisone, and hydrocortisone, and the glucose tolerance and plasma amino nitrogen concentrations followed for periods up to 48 hours. In rats with intact adrenals, or adrenalectomized at the time of evisceration, exogenous adrenal steroids produced no effect over peroids up to 6 hours. If adrenalectomy was performed 24 hours before evisceration, the steroids produced an accelerated rise of plasma amino nitrogen. When 24 or 48 hours had elapsed after simultaneous adrenalectomy and evisceration, the steroids caused similar effects and also decreased glucose tolerance. Insulin did not affect the qualitative nature of the response, although it reduced the concentrations of glucose and amino nitrogen. At high glucose and insulin loads, cortisone increased the glucose tolerance of adrenalectomized rats. These studies indicate that in the rat the protein and carbohydrate regulating effects of adrenal steroids can be produced in the absence of the liver.

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NOTES AND COMMENTS

CONCERNING THE ABSORPTION OF DESOXYCORTICOSTERONE TRIMETHYLACETATE FROM TISSUE SITES

It has been demonstrated that desoxycorticosterone trimethylacetate (DCT) differs from desoxycorticosterone acetate (DCA) in having a much longer duration of activity after single subcutaneous or intramuscular injections (Wieland, *et al.*, 1951; Gross and Tschoff, 1952; Swingle, *et al.*, 1952; Gaunt, *et al.*, 1952; Thorn, *et al.*, 1953; Sorkin and Soffer, 1953). In addition, at least in the rat, DCT exerted more total activity, mg. for mg., than did DCA (Gaunt, Leathem, Howell and Antonchak, 1952).

There are various possible reasons to account for these differences between DCA and DCT. One is that DCT might escape to some degree the hepatic inactivation or other metabolic fates to which DCA is subject (cf. Eversole and Gaunt, 1943). That possibility has not been definitely eliminated as indicated below.

Another obvious and likely possibility is that DCT is more slowly absorbed from tissue sites than DCA. That is shown here to be the case.

RESULTS

Experiments were first undertaken to determine the effects of the subcutaneous and intramesenteric implantation of single 15 mg. pressed pellets of DCT. They were implanted at the time of adrenalectomy in immature male rats. In contrast to the effects of DCT when administered in microcrystalline suspension or oil solution, it was noted that the pellets were weak or inactive in maintaining life of adrenalectomized rats. Mean survival of groups of 12 animals was as follows: untreated controls, 7 days; subcutaneous DCT pellets, 11 days; intramesenteric pellets, 6.7 days. The fact that pellets implanted in the mesentery may have been slightly less active than those at subcutaneous sites, suggests that DCT, like DCA, may be partially inactivated in the liver. On the other hand, no differences in survival were noted when microcrystalline suspensions of DCT were injected intrasplenically or subcutaneously. Consequently, the possible role of hepatic inactivation remains obscure.

The probable reason, however, for the lack of clearly significant activity of DCT pellets at any site was that there was no apparent absorption of steroid from the pellets during the short periods of survival.

To study this aspect of the problem further, 15 mg. pellets of DCA or DCT, of similar size and shape, were implanted subcutaneously into normal male rats weighing approximately 60 gm. each. After either 20 or 40 days, the pellets were removed and dried to constant weight. During these intervals appreciable absorption occurred of the DCA but not the DCT pellets (Table 1). It is possible that the apparent total lack of absorbability of DCT was due to an artifact in which the weight of dried surrounding tissue elements equalled that of absorbed steroid but at best absorption was negligible.

These observations, together with previous ones, indicate that the insolubility of DCT in tissues is such that it can be absorbed in effective amounts only if given in solution or microcrystalline suspension but not from small pellets of low exposed surface area.

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TABLE 1. ABSORPTION OF DESOXYCORTICOSTERONE ACETATE AND TRIMETHYLACETATE PELLETS IN INTACT RATS

(Mean values from 5 animals per test group)

Steroid	Pellet weight (mg.)		
	Original	After 20 days	After 40 days
DCA	15.8 16.6	12.4 —	— 11.2
DCT	15.9 15.8	16.0 —	— 15.8

SUMMARY

There was at most only a negligible absorption from 15 mg. pellets of desoxycorticosterone trimethylacetate implanted subcutaneously in rats for periods up to 40 days. Measurable absorption of DCA pellets occurred under the same conditions. The slow absorption of DCT probably accounts largely or perhaps entirely for the fact that when given in solution or suspension this steroid has a much greater duration of activity than DCA.

ADDENDUM

It has been observed subsequently that when six 15-mg. pellets of DCT were placed at separate subcutaneous sites, there was sufficient absorption to maintain life and growth in adrenalectomized rats for periods longer than 15 weeks.

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EFFECTS OF HORMONES ON THE COENZYME A CONCENTRATION IN RAT LIVER[1]

This investigation deals with changes in the coenzyme A (Co-A) concentration in the livers of rats following hypophysectomy and hormonal therapy. Tabachnick and Bonnycastle (1953) previously reported that Na-L-thyroxine pentahydrate increased liver Co-A in the thyroidectomized rat.

METHODS

Adult hypophysectomized female rats of the Long-Evans strain were used. All treatments were given for 3 successive days, beginning one week after the operation. Cortisone acetate, growth hormone, L-thyroxine and several combinations of the hormones were employed, and in some instances, calcium pantothenate was also given. Growth hormone was injected intraperitoneally, the other substances, subcutaneously. The rats were starved 24 hours before autopsy. Coenzyme A concentrations were determined by the acetylation method of Kaplan and Lipmann (1948), and are reported as Lipmann units per gram of tissue, wet weight.

RESULTS

Hypophysectomy induced a marked decrease in Co-A concentration in the liver.[2] Injections of cortisone acetate increased the Co-A concentration, the effect being maximal at the low dose level. Adequate doses of growth hormone substantially increased the coenzyme level in the hypophysectomized rats. L-thyroxine was as effective as cortisone (Table 1).

The failure of these hormones to restore the Co-A concentration to normal in the livers of hypophysectomized rats, and the fact that concomitant administration of these hormones are known to act synergistically in other responses, suggested experiments to determine if possible synergistic effects could be demonstrated on Co-A levels. When various combinations of cortisone, growth hormone and L-thyroxine were injected concomitantly, no evidence of a definite synergistic effect was observed (Table 1). The effect of growth hormone plus L-thyroxine was slightly greater than additive, but in none of these experiments was Co-A elevated to the normal level. It thus appears that more than one hormone can increase the Co-A concentration in the liver, and it is suggested that the mechanism of action may be different for each hormone.

Recognizing that inadequate availability of pantothenic acid in hypophysectomized rats (lower food consumption, poor absorption) might be a factor in the observed low Co-A values, the vitamin was injected into a group of such rats. This resulted in a 47 per cent increase in liver Co-A which indicates that it is possible to increase this coenzyme without the necessary intervention of these hormones. The effect of growth hormone and pantothenate given concomitantly was additive (Table 1).

Since Lotspeich (1950) showed that growth hormone treatment in normal rats increased the pantothenic acid requirement, a study was made on the effect of this hormone on the Co-A levels in normal rat livers. Groups of 3 rats were killed 6, 12 and 24 hours after a single injection of 0.375 mg. of growth hormone. The average Co-A units

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[1] This research was supported in part by a grant from the Nutrition Foundation, Inc., New York, N.Y., and by a grant from the Faculty Research Grant Committee of Cornell University.

[2] Diphosphopyridine nucleotide (DPN) concentration in the liver was decreased 20% (in contrast to 57% decrease in Co-A) following hypophysectomy and was returned to normal by low doses of growth hormone and cortisone. L-thyroxine further depressed the DPN level.

per gram fresh liver were 168, 177 and 155 respectively, as compared to the initial level of 215 units. The paradoxical action of growth hormone increasing the Co-A concentration in hypophysectomized rats and lowering it in normal rats is difficult to reconcile at present.

Although the hypophysectomized animals were fasted for 24 hours before killing, this appeared to be unnecessary as subsequent experiments indicated. Ten hypophysectomized rats fed to autopsy had liver Co-A concentrations of 73 units (58–89), which can be compared to 94 units in the fasted controls. The effect of feeding versus fasting on the liver Co-A was also made in normal (Sprague-Dawley) rats. The average Co-A units (for groups of 6 rats) found at various periods after fasting were as follows: 0 hours fasting, 151 (129–172); 24 hours fasting, 157 (135–168); and 4 days fasting, 163 (125–182). Thus fasting in normal rats did not influence Co-A concentrations, but it appears that there is a strain difference in basic Co-A levels.

These results indicate the importance of studying coenzymes in target organs affected by hormones in an analysis of the hormonally controlled biochemical reactions, and particularly those which are Co-A dependent.

SUMMARY

Hypophysectomy decreased coenzyme A concentration in rat liver. Cortisone acetate, growth hormone, L-thyroxine and calcium pantothenate injections increased the Co-A level in hypophysectomized rats. No synergistic action was observed with combined hormone treatments. Growth hormone decreased liver Co-A levels in normal rats.

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TABLE 1. COENZYME A CONCENTRATION IN HYPOPHYSECTOMIZED RAT LIVERS UNDER VARIOUS HORMONAL TREATMENTS

Treatment*	No. of rats	Coenzyme A units per gram tissue	
		Range	Average
None (Intact rats)	10	195–238	215
None (Hypsect.)	17	53–116	94
Cortisone (2 mg.)	11	116–132	124
Cortisone (5 mg.)	10	102–154	123
Thyroxine (150 μg.)	11	102–165	124
Growth Hormone (0.375 mg.)	18	56–135	108
Growth Hormone (1.5 mg.)	10	122–208	176
Growth Hormone+Cortisone	10	111–139	128
Growth Hormone+Thyroxine	9	152–188	163
Growth Hormone+Cortisone+Thyroxine	9	165–188	178
Pantothenate (5 mg.)	6	122–148	139
Growth Hormone+Pantothenate	6	152–182	164

* Where combinations of the hormones were administered, only the smaller doses were used.

Acknowledgment

The growth hormone (prep. K40105R) was donated by the Armour Research Lab., Chicago, Illinois, and the cortisone acetate by the Schering Corporation, Bloomfield, New Jersey.

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AWARDS OF THE ENDOCRINE SOCIETY

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

MEDAL OF THE ENDOCRINE SOCIETY

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts); 1954—Dr. Isador N. Rosenberg. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-four months of the date of the award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship was established in 1947. It is designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. The Fellowship is awarded on alternate years (1955, 1957, etc.) and the stipend which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the

qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree or candidates for either of these degrees are eligible for appointment.

Applicants must submit the following information:

(1) Evidence of scientific ability as attested by studies completed or in progress.
(2) Recommendations from individuals familiar with the candidate and his work.
(3) A proposed program of study.
(4) Acceptance of the individual by the head of the department in which the Fellowship will be held.
(5) A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

Nominations

Nominations for the Medal of The Endocrine Society, the Ciba Award, and the Ayerst, McKenna and Harrison Fellowship may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the office of the Secretary, Suite 319, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than November 1, 1954. The Awards Committee will meet in November and notice of awards to successful applicants will be made not later than December 1, 1954.

GROWTH HORMONE SYMPOSIUM

A 3-day International Symposium on the HYPOPHYSEAL GROWTH HORMONE, ITS NATURE AND ACTIONS is being sponsored by the Henry Ford Hospital and the Edsel B. Ford Institute for Medical Research. The Symposium will be held in the New Clinic Building Auditorium of the Henry Ford Hospital, October 27, 28 and 29, 1954.

Formal presentations and discussions will cover the following aspects of growth hormone investigation: bioassay, preparation and physicochemical properties of growth hormone; effects of growth hormone on certain structures such as the mammary gland, skeletal system, connective tissue, Islets of Langerhans, kidneys, gastrointestinal tract and tissue cultures; growth hormone and energy sources; the effect of growth hormone on cellular systems; the influence of growth hormone on lactation; and its metabolic effects in man.

Interested persons should write the Program Committee, Henry Ford Hospital, Detroit 2, Michigan. Invitations will be sent to as many persons as possible.

SYMPOSIUM ON ADRENAL FUNCTION IN INFANTS AND CHILDREN

A Symposium on Adrenal Function in Infants and Children will be held at the State University of New York, Upstate Medical Center in Syracuse, November 3 and 4, 1954. The Symposium is sponsored by the Department of Pediatrics, State University of New York, College of Medicine in Syracuse and by the M. and R. Laboratories. For further information address: Office of the Assistant Dean, Postgraduate Medical Education, 766 Irving Ave., Syracuse 10, N. Y.

MEDICAL RESEARCH FELLOWSHIPS

The Division of Medical Sciences of the National Academy of Sciences-National Research Council is accepting applications for postdoctoral research fellowships for 1955–1956. These awards are designed to offer research experience for promising individuals who look forward to investigative careers, and not to provide practical experience in the clinical field. Ordinarily Fellowships are not granted to persons over thirty-five years of age. The following programs are announced:

Fellowships in Cancer Research are awarded by the American Cancer Society on recommendation of the Committee on Growth of the Division

of Medical Sciences. Awards are available for study in all branches of the biological, chemical and physical sciences and of clinical investigation applicable to the study of growth, typical or malignant. Citizens of the United States are eligible.

British-American Exchange Fellowships in Cancer Research also are awarded by the American Cancer Society upon recommendation by the Committee on Growth. They are offered to citizens of the United States for advanced study in Great Britain in specialized fields pertaining to the problem of growth. Similar fellowships are awarded by the British Empire Cancer Campaign to young British scientists for research in the United States.

Fellowships in the Medical Sciences supported by The Rockefeller Foundation and by The Lilly Research Laboratories, are administered by the Medical Fellowship Board of the Division. Fellows are expected to devote themselves to research in the basic medical sciences. The Fellowships administered for The Rockefeller Foundation are open to citizens of the United States and Canada; the Lilly Fellowships only to citizens of the United States.

Fellowships in Tuberculosis are also administered by the Medical Fellowship Board under a grant from the National Tuberculosis Association. These awards are designed to promote the development of investigators in fields related to tuberculosis. They are open to citizens of the United States who are graduates of American schools.

Fellowships in Radiological Research are administered for the James Picker Foundation by the Division's Committee on Radiology. The Foundation has expressed particular interest in the support of candidates who propose to carry on research oriented toward the diagnostic aspects of radiology. Appointments are not limited to citizens of the United States.

Applications for 1955–1956 under any of these programs must be postmarked on or before *10 December 1954*. Fellowships are awarded in the early Spring. Complete details and application blanks may be obtained from the *Fellowship Office, National Academy of Sciences-National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D.C.*